Letter to the Editor

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Potential interference of hydroxychloroquine-glucuronide metabolite on therapeutic drug monitoring of hydroxychloroquine using a mass spectrometry detector

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To the Editor,

Due to a new coronavirus the world has been experiencing an infectious disease for several weeks. Coronavirus disease (COVID-19) causes severe acute respiratory syndrome in some people, with an unexpected number of deaths. In this pandemic context, a European clinical trial named DisCoVeRy has started to test four experimental treatments including several drugs [1]. One of the modalities' treatment includes standard of care associated with hydroxychloroquine (HCQ) administration. HCQ is first known for the prevention and the treatment of malaria and for the treatment of several autoimmune diseases too [2–4]. For DisCoVeRy, HCQ is administered orally or by using a nasogastric tube, and plasmatic quantification is programmed on days 1, 3, 5, 8 and 11 [1]. This treatment may also be proposed on a compassionate basis for patients not included in clinical trials, associated with possible dose adjustment according to plasma concentration [5]. Several analytical methods for the quantification of HCQ and its three major metabolites (monodesethylhydroxychloroquine, desethylchloroquine and bisdesethylchloroquine) have already been published [2–4]. However, few data are available concerning the phase II hydroxychloroquine-glucuronide (HCQ-Glu) metabolite [6, 7]. This metabolite does not appear to have any pharmacological

activity. Under certain analytical conditions, it can lead to error in the quantification of HCQ. This present letter focuses on this point.

In the context of pandemic disease and the DisCoVeRy clinical trial, few technical staff and many samples, we selected a short chromatography and rapid sample preparation, in order to reduce the delay for results. An Acquity™ UPLC system coupled with a Xevo™ TQ MS (Waters®, USA) triple quadrupole MS equipped with an electrospray Z-spray source™ was used. HCQ was monitored in positive ion mode and multiple reaction monitoring (MRM) mode with three transitions m/z 336 → 247 (used for quantification), m/z 336 → 158 and m/z 336 → 179 (both used as qualifier ions), the collision energies were 20 eV, 24 eV and 40 eV, respectively. The optimal signal-to-noise ratio for HCQ quantification was observed with a cone voltage set at 30 V. Samples were separated on an Acquity™ UPLC BEH C18 1.7 μ m 2.1 × 150 mm (Waters®, USA) at +45 °C with a flow rate at 420 μ L/min. The composition of the mobile phase and gradients used are presented in Figure 1. Ostro™ plate (Waters®, USA) was used for sample preparation as follows: 50 μ L of sample was mixed with 150 μ L of acetonitrile containing labelled chloroquine-d5 as internal standard and 1% formic acid. Eluate was diluted in 400 μ L of deionized water and 10 μ L was injected. A liquid/liquid extraction (LLE) with 1 mL of sample, 3.5 mL of chlorobutane and 100 μ L of 25% ammonia, was also performed. This LLE extracted apolar compounds such as HCQ and removed polar compounds such as glucuronide metabolite.

During the development of the UPLC-MS/MS assay, an unsymmetrical “shoulder” peak for HCQ with a retention time at 1.03 and 1.14 min was observed (Figure 1). This peak shape was only observed from patient samples after extraction with an Ostro™ plate but not with LLE. Whatever the extraction process used no “shoulder” peak was observed from standard or quality control samples. Modification of the liquid chromatography (LC) gradient enabled the separation of the two peaks with retention times at 1.98 and 2.12 min, respectively (Figure 1).

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Figure 1: Chromatograms obtained with the transition m/z 336 $\rightarrow$ 247 used for hydroxychloroquine quantification on Xevo TQ-MSTM LC-MS/MS device.

Chromatograms A and B were from patient sample and C from quality control (0.2 mg/L). In each case the gradient used was presented under the corresponding chromatograms. Phase mobile composition, %A: formate buffer (5 mM) and %B: formic acid (0.1%) acetonitrile.

According to the gradient used, the peak with a retention time at 1.14 and then at 2.12 min corresponded to HCQ.

An unlikely hypothesis, was that the two peaks could be the R and S enantiomers of HCQ. This hypothesis was rejected because the analysis of a racemic mixture of HCQ showed only one chromatographic peak.

A second hypothesis was that the peak at retention time 1.03 min and then 1.98 min, corresponds to HCQ-Glu. To test this hypothesis, plasma samples from patients were deconjugated with β -glucuronidase overnight at 37 °C (type IX-A from *Escherichia coli* – Merck®, USA). Analysis of hydrolysis samples showed only one peak at 2.12 min corresponding to HCQ with a peak area approximately equal to the sum of the area of peaks of HCQ-Glu and HCQ before hydrolysis. We concluded that fragmentation of HCQ-Glu and the liberation of HCQ in the ion source leads to detecting two peaks in the MRM chromatogram of the parent drug. We varied the cone voltage from 10 to 50 V (Figure 2). At 10 V only the HCQ peak was observed, which means that the HCQ-Glu is not fragmented at this voltage. For higher cone voltage, the higher the voltage the larger the area of the peak corresponding to the HCQ-Glu. From samples patients, HCQ-Glu peak was observed with varying intensities according to the time sample and patients. Thus, the impact on therapeutic drug monitoring (TDM) of HCQ will vary according to the samples. Note that a partial *in vitro* deglucuronidation could lead to overestimation of HCQ concentrations. However, several studies have shown no

degradation of glucuronide-metabolites in plasma under conventionally used storage conditions [8, 9].

Tests were also carried out on the LC Ultimate 3000TM system coupled with a Q-Exactive Plus OrbitrapTM mass spectrometer with an ion-source HESI-II Ion MaxTM (ThermoFisher Scientific®, Germany). LC conditions were identical to those previously described. Analyses were performed by using the full scan and parallel reaction monitoring mode. With this ion source, although the spray current was increased, no fragmentation of HCQ-Glu was observed. The exact mass of the peak at 1.98 min (m/z 512.2164) corresponds to that of HCQ-Glu and its fragmentation leads to detecting daughter ions at m/z 336.1833 (corresponding to $[M+H]^+$ ion of HCQ), and at m/z 247.0994.

Other analysis performed on an LC-DAD-MS (H-ClassTM, Waters®) with QDATM detector (single-quadrupole mass spectrometry [MS] and electrospray ionization [ESI] source). Chromatographic conditions separated HCQ-Glu and HCQ (isocratic separation with ammonium acetate buffer [5 mM, pH 2.5]/acetonitrile [86/14; v/v] on a dC18 AtlantisTM 3 μ m, [150 mm $\times$ 3 mm i.d.] column). The MS signal at m/z 336 also shows both compounds demonstrating the fragmentation of HCQ-Glu with this device, and the peak area was related to the cone voltage.

Finally, the data from this study indicate that interference from a metabolite such as the glucuronide conjugate can be observed even with an approach as selective as LC-MS/MS in the MRM mode. We have shown that,

Figure 2: Chromatogram obtained with the transition m/z 336 $\rightarrow$ 247, depending on the cone voltage applied on Z-ion source. The retention time of peaks 1 and 2 correspond to hydroxychloroquine-glucuronide and hydroxychloroquine, respectively. CV, cone voltage (V).

depending on the type of MS source, the chromatographic conditions, the tuning parameters, HCQ-Glu can interfere with the quantification of HCQ leading to affect TDM results. The ion-source fragmentation of the glucuronide metabolite and the formation of the parent drug have already been described for mycophenolic acid and posaconazole [8, 10]. Solutions to get around this issue will be therefore to use more selective extraction such as

LLE but with a time-consuming protocol, or the optimization of chromatographic separation and selection of MS parameters.

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