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Estimation of the uncertainty of values assigned to calibration materials prepared *in-house*: An example for hydroxychloroquine calibrators in blood-hemolysate-based matrix

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ABSTRACT

Background: Hydroxychloroquine is an antimalarial drug that has been prescribed for the treatment of patients with COVID-19 infection. To assist in clinician decision-making, several clinical laboratories have developed and validated measurement procedures *in-house* based on HPLC or HPLC-MS/MS to measure the mass concentration of hydroxychloroquine in different biological fluids. In these cases, laboratories produce their calibration materials but rarely estimate the measurement uncertainty of their assigned values. Thus, we aimed to show how this uncertainty can be calculated, using the preparation of hydroxychloroquine calibrators in blood-hemolysate-based matrix as an example.

Methods: A bottom-up approach was used to estimate the uncertainty related to the values assigned to end-user calibration materials prepared *in-house*. First, a specification of the measurand and a measurement equation were proposed. Then, different sources of uncertainty related to the preparation of hydroxychloroquine calibration materials were identified and quantified. Afterwards, the combined uncertainty was calculated using the *law for the propagation of uncertainty* resulting in the final expanded uncertainty.

Results: In this study, the most significant source of uncertainty was that associated with the hydroxychloroquine's reference material mass obtained via balance, while the smallest contribution was from the uncertainty associated with the hydroxychloroquine reference material purity.

Conclusions: A simple procedure to estimate the measurement uncertainty of values assigned to calibration materials is presented here, which would be easy to implement in clinical laboratories. Also, it could be put into

Abbreviations: HCQ, hydroxychloroquine; RM, reference material; MU, measurement uncertainty; $u(\mathrm{cal})$, measurement uncertainty related to the values assigned to the end-user calibration materials; $u(m)$, uncertainty associated with the HCQ reference material mass obtained via balance; $u_{rel}(\mathrm{m})$, relative uncertainty associated with the HCQ reference material mass obtained via balance; $u(p)$, uncertainty associated with the purity of the reference material; $u_{rel}(\mathrm{p})$, relative uncertainty related to the purity of the HCQ reference material; $u(\mathrm{V_f})$, uncertainty related to the internal volume of the flask; e, volumetric flask accuracy; $u(\mathrm{T, flask})$, uncertainty associated with the volumetric flask volume variation due to room temperature fluctuations; $u(V_{flask})$, uncertainty related to the volumetric flask volume used to prepare the HCQ stock solution; $u_{rel}(\mathrm{V_{flask}})$, relative uncertainty associated with the volumetric flask volume used to prepare the HCQ stock solution; $u(\mathrm{V_p})$, uncertainty related to the pipette calibration; $u(\mathrm{T, pipette})$, uncertainty associated with the pipette volume variation due to room temperature fluctuations; $u(\mathrm{pipette})$, uncertainty associated with the pipetted volume; $u(V_{p,stock})$, uncertainty related to the volume of stock solution pipetted to prepare the corresponding HCQ working standard solution; $u_{rel}(\mathrm{V_{p.stock}})$, relative uncertainty related to the volume of stock solution pipetted to prepare the corresponding HCQ working standard solution; $u(V_{p.water})$, uncertainty associated with the volume of LC/MS-grade water pipetted to prepare the corresponding working standard solution; $u_{rel}(\mathrm{V_{p.water}})$, relative uncertainty associated with the volume of LC/MS-grade water pipetted to prepare the corresponding HCQ working standard solution; $u(V_{p.ws})$, uncertainty associated with the volume of the corresponding working standard solution pipetted to prepare the appropriate calibration material; $u_{rel}(\mathrm{V_{p.ws}})$, relative uncertainty associated with the volume of the corresponding working standard solution pipetted to prepare the appropriate HCQ calibration material; $u(V_{p.blood})$, uncertainty related to the volume of the corresponding drug-free hemolysate solution pipetted to prepare the appropriate HCQ calibration material; $u_{rel}(\mathrm{V_{p.blood}})$, relative uncertainty related to the volume of the corresponding drug-free hemolysate solution pipetted to prepare the appropriate HCQ calibration material; cHCQ, mass concentration of hydroxychloroquine in blood-hemolysate-based matrix end-user calibration material value; $u_c(\mathrm{cHCQ})$, combined standard uncertainty related to the cHCQ; $u_{c,rel}(\mathrm{cHCQ})$, relative combined standard uncertainty related to the cHCQ; $U(\mathrm{cHCQ})$, expanded uncertainty associated with the cHCQ.

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practice for other pharmacological quantities measured by *in-house* HPLC or HPLC-MS/MS procedures commonly used in clinical laboratories.

1. Introduction

Several months ago, hydroxychloroquine (HCQ) was prescribed extensively across the world among patients with COVID-19 infection, considering its *in-vitro* effect against the SARS-CoV-2 virus. At present, based on different observational studies and clinical trials, international organisations or agencies do not recommend prescribing HCQ for patients with COVID-19 infections. Despite this, the efficacy and safety of HCQ remain unclear, and some healthcare systems continue to administer this drug to COVID-19 patients [1–3].

During the "first wave" of COVID-19 crisis, several clinical laboratories developed and validated measurement procedures based on HPLC or HPLC-MS/MS to measure the mass concentration of HCQ in different body fluids [4,5] to help clinicians improve treatment adherence, adjust doses, and minimise the risk of short and long-term side effects. It was necessary to develop and validate these procedures *in-house* because no available CE-marked or commercial reagent kits existed.

Part of the *in-house* laboratory development procedure is preparation of calibration materials, from particular reference material (RM), and validation of the calibration curve over a previously specified measurement interval to test the responses of the instrument concerning the biological quantity values [6,7]. In these specific cases, when the clinical laboratory produces the calibration materials, it is responsible for assigning their values and estimating the measurement uncertainty (MU).

To guarantee that the calibration materials are fit for their intended purpose, the laboratory should prepare them in the same matrix of the patient samples from a RM of known identity and purity. This RM should also have, whenever possible, assigned values traceable to the SI through a stated reference, i.e., a higher-order metrological RM or reference measurement procedure included in the JCTLM database [8]. In addition, the laboratory should estimate the MU related to the values assigned to calibration materials taking into account all information used to their preparation, and statistically combining the uncertainties associated with each of the value assignment steps [9–11].

In this study, we aimed to provide a proposal to estimate the MU related to the values assigned to end-user calibration materials produced *in-house* using, as an example, the preparation of HCQ calibrators in blood-hemolysate-based matrix.

2. Material and methods

2.1. Chemicals and reagents

Reference material of HCQ sulfate (purity 99.87%; Cat. n. LGCFOR0764.00) was purchased from LGC Standards (Middlesex, UK). LC-MS-grade methanol and water were supplied by Merck Millipore Group (Darmstadt, Germany).

Blood-hemolysate-based matrix calibration materials were prepared using a human blood pool. Blood was collected in EDTA-K_3 tubes (Vacuette, Kremsmünster, Austria) from patient donors at our hospital. We performed a hemolysis procedure as described in Grote-Koska *et al.* [12] to minimize possible problems regarding the viscosity and homogeneity.

2.2. Equipment

The following equipment was used:

- Radwag AS 60/220.R2 analytical balance from Radwag Wagi Elektroniczne (Radom, Poland). Uncertainty indicated by the accredited calibration laboratory certificate was (5.1 ± 0.2) mg ($k = 2$).
- Nichipet® EX II adjustable (1–10) μL micropipette (pipette A), Nichipet® EX II adjustable (20–200) μL micropipette (pipette B), and Nichipet® EX II adjustable (100–1000) μL micropipette (pipette C) from Nichiryo Co Ltd. (Koshigaya-shi, Saitama, Japan). Expanded uncertainties ($k = 2$) indicated by the accredited calibration laboratory certificate were: (5.01 ± 0.14) μL and (10.0 ± 0.2) μL for pipette A; (20.0 ± 0.5) μL, (50.0 ± 0.8) μL, (100.0 ± 1.2) μL, and (200.0 ± 2.3) μL for pipette B; and (1000 ± 4) μL for pipette C.
- 50-mL BLAUBRAND® volumetric flask USP certified (BRAND GMBH + CO KG, Wertheim, Germany). According to the manufacturer's data, the 50-mL volumetric flask inaccuracy was 0.05 mL.

2.3. Calibration materials preparation

A stock solution of 100 mg/L HCQ in a 50 mL-volumetric flask was produced by dissolving 5.0 mg of the HCQ's RM in methanol. Then, eight working standards of 1-mL at values close to 0.50, 1.0, 2.0, 4.0, 6.0, 10.0, 15.0 and 20.0 mg/L were prepared by pipetting the corresponding volumes of stock solution into water. After that, 100 μL-aliquots of calibration materials were made (close to 50.0, 100.0, 200.0, 400.0, 600.0, 1000, 1500 and 2000 μg/L) diluting the working standards in drug-free hemolysate solution in a 1:9 ratio (see Table 1). Finally, calibration materials were stored protected from light at (–75 ± 3) °C.

Note that we avoided precipitation of the calibration materials by not directly combining the stock and hemolysate solutions.

2.4. Measurement uncertainty estimation

A *bottom-up* approach was proposed to estimate the MU related to the values assigned to the end-user calibration materials, $u(\mathrm{cal})$. The $u(\mathrm{cal})$ estimation was based on the following steps [13]:

2.4.1. Measurand specification

Measurand was defined as the mass concentration (in μg/L) of HCQ calibration materials prepared in a blood-hemolysate-based matrix (cHCQ) as described above, and values were assigned using the following measurement equation:

$$\mathrm{cHCQ} = \frac{10^6 \cdot m \cdot p}{V_{\mathrm{flask}}} \cdot \frac{V_{\mathrm{p,stock}}}{\left(V_{\mathrm{p,stock}} + V_{\mathrm{p,water}}\right)} \cdot \frac{V_{\mathrm{p,ws}}}{\left(V_{\mathrm{p,ws}} + V_{\mathrm{p,blood}}\right)} \quad (1)$$

where:

10^6 conversion factor from [mg/mL] to [μg/L]

m mass of the HCQ's RM weighed into the balance [mg]

p purity of the HCQ's RM given as mass fraction [g/g]

V_{flask} volume of the volumetric flask used to prepare the HCQ's stock solution in LC/MS-grade methanol [mL]

$V_{\mathrm{p,stock}}$ volume of stock solution pipetted to prepare the corresponding HCQ's

working standard solution [μL]

$V_{\mathrm{p,water}}$ volume of LC/MS-grade water pipetted to prepare the corresponding

HCQ's working standard solution [μL]

$V_{\mathrm{p,ws}}$ volume of the corresponding working standard solution pipetted to prepare the appropriate HCQ calibration material [μL]

$V_{\mathrm{p,blood}}$ volume of the corresponding drug-free hemolysate solution pipetted to prepare the appropriate HCQ calibration material [μL]

2.4.2. Identification of sources of uncertainty

We used the *cause and effect* diagram to identify the most relevant sources of uncertainty (see Fig. 1). The primary sources of uncertainty considered were those related to the mass obtained via balance, the RM's purity, the volume of the flask used to produce the stock solution, and the volumes of the pipettes used to prepare working standards and end-user calibration materials.

2.4.3. Estimation of standard uncertainties

2.4.3.1. Mass. The uncertainty associated with the HCQ's RM mass obtained via balance, $u(m)$, was estimated as:

$$u(m) = \frac{U(\text{balance})}{2} \quad (2)$$

The expanded uncertainty of the balance, $U(\text{balance})$, provided by the accredited calibration laboratory certificate, included three primary sources of uncertainty: the repeatability, the readability (digital resolution) of the balance scale, and the contribution due to the uncertainty in the calibration function (linearity) of the scale range.

2.4.3.2. Purity. According to the certificate of analysis of the HCQ's RM, its purity (p) was calculated as:

$$p = (1 - \text{KF}_{\text{WC}}) \cdot p_{\text{HPLC}} \quad (3)$$

where KF_{WC} is the RM water content obtained using the Karl-Fisher titration method (KF = 0.0009 ± 0.0002 with a $k = 2$); and p_{HPLC}, the purity value obtained by a specific HPLC method (p_{HPLC} = 0.9996 ± 0.0002 with a $k = 2$).

From the p measurement equation (Eq.3), and applying the GUM *law for the propagation of uncertainty* [13], the standard uncertainty associated with the RM's purity, $u(p)$, was calculated as:

$$u(p) = p \cdot \sqrt{\left[\frac{u(\text{KF}_{\text{WC}})}{(1 - \text{KF}_{\text{WC}})}\right]^2 + \left[\frac{u(p_{\text{HPLC}})}{p_{\text{HPLC}}}\right]^2} \quad (4)$$

Note that all units are 1 instead of %.

2.4.3.3. Volumetric flask volume. The volume of the stock solution delivered by the volumetric flask is subject to two major sources of uncertainty:

a) The uncertainty related to the internal volume of the flask, $u(\text{V}_\text{f})$. The $u(\text{V}_\text{f})$ was calculated using the volumetric flask inaccuracy (e) in mL given by the manufacturer, and assuming a type-B-triangular distribution:

$$u(\text{V}_\text{f}) = \frac{e}{\sqrt{6}} \quad (5)$$

b) The uncertainty associated with the volumetric flask volume variation due to room temperature fluctuations, $u(\text{T,flask})$. Note that the flask and solution temperatures can differ from the temperature at which the flask volume was calibrated (at 20 °C). Thus, the $u(\text{T, flask})$ was estimated assuming a type-B-rectangular distribution as:

$$u(\text{T, flask}) = \frac{\alpha \cdot \Delta T}{\sqrt{3}} \quad (6)$$

where α is the coefficient of volume expansion for methanol at 20 °C ($1.49 \cdot 10^{-3}$ 1/°C); and ΔT is the difference between the actual laboratory temperature and the temperature during the calibration of the volumetric flask (ΔT = 5 °C).

Thus, the two contributions described above were considered and combined to obtain the uncertainty related to the volumetric flask volume, $u(V_{\text{flask}})$:

$$u(V_{\text{flask}}) = \sqrt{u^2(\text{V}_\text{f}) + u^2(\text{T, flask})} \quad (7)$$

2.4.3.4. Pipette volumes. The uncertainty contributions considered for the pipetted volumes were those related to the pipette calibration, $u(\text{V}_\text{p})$, and the volume variations due to the room temperature fluctuations, $u(\text{T, pipette})$. The uncertainties associated with the different pipetted volumes, $u(\text{pipette})$, were calculated as:

$$u(\text{pipette}) = \sqrt{u^2(\text{V}_\text{p}) + u^2(\text{T, pipette})} \quad (8)$$

For the $u(\text{V}_\text{p})$, accredited calibration laboratory certificates include sources of uncertainty associated with repeatability and volume bias. In our case, accredited calibration laboratory indicates that the volume biases were not significant (they were lower than the maximum allowable relative bias previously established by them: ±1.0%), and it was not necessary to apply a volume bias-correction factor. So, the uncertainty related to the repeatability was only included as source of uncertainty of $u(\text{V}_\text{p})$.

For the $u(\text{T, pipette})$, it was estimated assuming a type-B-rectangular distribution:

$$u(\text{T, pipette}) = \frac{\alpha \cdot \Delta T}{\sqrt{3}} \quad (9)$$

and α is the coefficient of volume expansion at 20 °C for methanol ($1.49 \cdot 10^{-3}$ 1/°C), water ($2.1 \cdot 10^{-4}$ 1/°C) or blood ($3.0 \cdot 10^{-4}$ 1/°C); and ΔT is the difference between the actual laboratory temperature and the temperature during the calibration of the pipette (ΔT = 5 °C).

So, considering that four volumes ($V_{\text{p,stock}}$, $V_{\text{p,water}}$, $V_{\text{p,ws}}$, $V_{\text{p,blood}}$) were pipetted using different pipettes (pipettes 1, 2 and 3) to prepare the calibration materials (Table 1), their respective uncertainties were calculated as:

Table 1
Hydroxycloroquine mass-weighed, hydroxycloroquine reference material purity, and volumes used to assign the end-user calibration materials values.

Level	HCQ RM mass-weighed	HCQ RM Purity	Volumetric Flask volume	Pipetted volumes				Calibrator assigned value (μg/L)
	m(mg)	p(g/g)	V_{flask}(mL)	$V_{\text{p,stock}}$(μL)	$V_{\text{p,water}}$(μL)	$V_{\text{p,ws}}$(μL)	$V_{\text{p,blood}}$(μL)	
1	5.0	0.9987	50	5	995	10	90	49.9
2	5.0	0.9987	50	10	990	10	90	99.9
3	5.0	0.9987	50	20	980	10	90	199.7
4	5.0	0.9987	50	40	960	10	90	399.5
5	5.0	0.9987	50	60	940	10	90	599.2
6	5.0	0.9987	50	100	900	10	90	998.7
7	5.0	0.9987	50	150	850	10	90	1498.1
8	5.0	0.9987	50	200	800	10	90	1997.4

HCQ; hydroxycloroquine; RM, reference material; m, reference material mass-weighed into the balance; p; purity of the HCQ reference material; V_{flask}, volumetric flask volume used to prepare the HCQ stock solution; $V_{\text{p.stock}}$, volume of stock solution pipetted to prepare the corresponding HCQ working standard solution; $V_{\text{p.water}}$, volume of LC/MS-grade water pipetted to prepare the corresponding HCQ working standard solution; $V_{\text{p.ws}}$, volume of the corresponding working standard solution pipetted to prepare the appropriate HCQ calibration material; $V_{\text{p.blood}}$, volume of the corresponding drug-free hemolysate solution pipetted to prepare the appropriate HCQ calibration material.

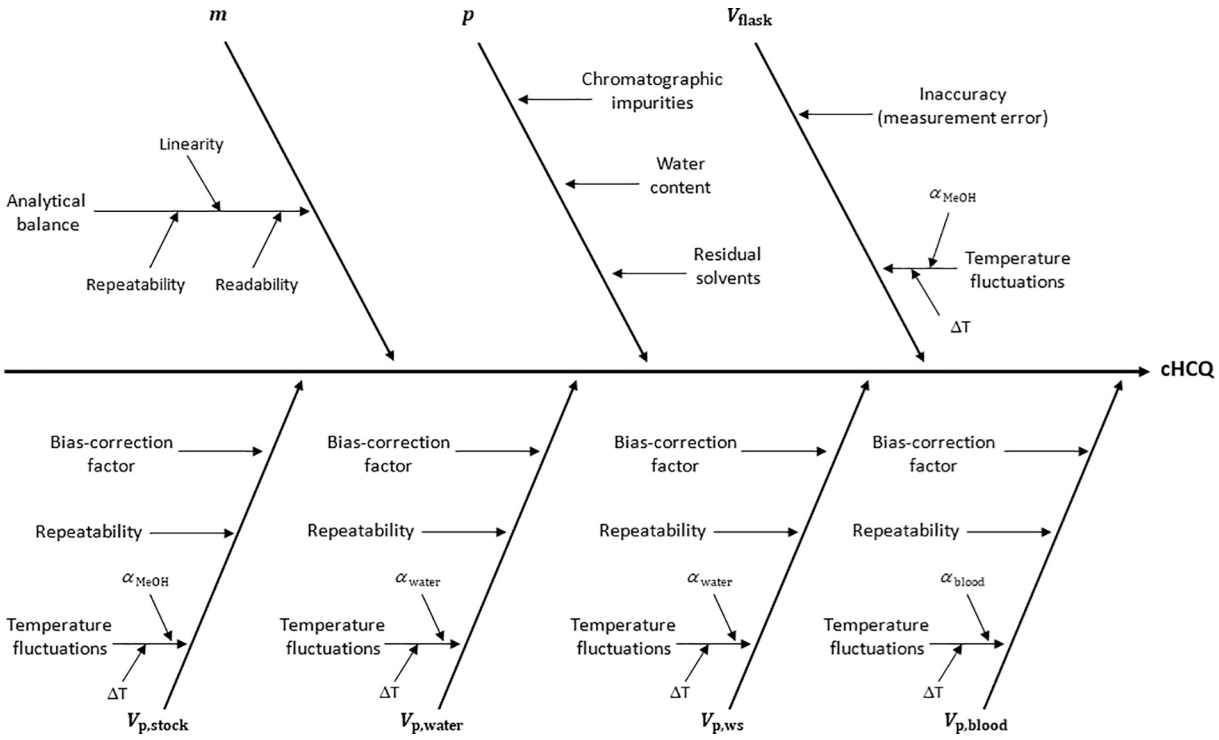

Fig. 1. Cause and effect diagram of the most relevant measurement uncertainty sources for the preparation of hydroxychloroquine end-user calibration materials. HCQ, hydroxychloroquine; cHCQ, mass concentration (in μg/L) of HCQ calibration material prepared in a blood-hemolysate based-matrix; m, HCQ reference material mass-weighed into the balance; p, purity of the HCQ reference material; V_{flask}, volumetric flask volume used to prepare the HCQ stock solution;α_{MeOH}, coefficient of volume expansion for methanol at 20 °C; ΔT, difference between the actual laboratory temperature and the temperature during the calibration of the volumetric flask; $V_{p,stock}$, volume of stock solution pipetted to prepare the corresponding HCQ working standard solution; $V_{p,water}$, volume of LC/MS-grade water pipetted to prepare the corresponding HCQ working standard solution; α_{water}, coefficient of volume expansion for water at 20 °C; $V_{p,ws}$, volume of the corresponding working standard solution pipetted to prepare the appropriate HCQ calibration material; $V_{p,blood}$, volume of the corresponding drug-free hemolysate solution pipetted to prepare the appropriate HCQ calibration material;α_{blood}, coefficient of volume expansion for blood at 20 °C.

$$u(V_{p,stock}) = \sqrt{u^2(V_{p1\ or\ p2}) + u^2(\mathrm{T},\ \text{pippete}\ \ 1\ \ \text{or}\ \ 2)} \tag{10}$$

$$u(V_{p,water}) = \sqrt{u^2(V_{p3}) + u^2(\mathrm{T},\ \text{pippete}\ \ 3)} \tag{11}$$

$$u(V_{p,ws}) = \sqrt{u^2(V_{p1}) + u^2(\mathrm{T},\ \text{pippete}\ \ 1)} \tag{12}$$

$$u(V_{p,blood}) = \sqrt{u^2(V_{p2}) + u^2(\mathrm{T},\ \text{pippete}\ \ 2)} \tag{13}$$

2.4.4. Calculation of the combined uncertainty

For a quantity y which depends on several uncorrelated measured quantities x_1, x_2, x_3, …, x_n, the combined standard uncertainty, $u_c(y)$, can be obtained using the GUM *law for the propagation of uncertainty* by combining estimates of the various x's [13] as follows:

$$u_c(y) = \sqrt{\sum_{i=1}^{n}\left(\frac{\partial y}{\partial x_i}\right)^2 \cdot u^2(x_i)} \tag{14}$$

where the partial derivatives ($\frac{\partial y}{\partial x_i}$) are the so-called *sensitivity coefficients* [13].

Therefore, considering Eq. (1) and applying the general format as described in Eq. (14), the combined standard uncertainty,$u_c(\mathrm{cHCQ})$, was calculated using the following equation (Eq. (15)):

$$\begin{aligned} u_c^2(\mathrm{cHCQ}) = {} & \left(\frac{\partial \mathrm{cHCQ}}{\partial m}\right)^2 \cdot u^2(m) + \left(\frac{\partial \mathrm{cHCQ}}{\partial p}\right)^2 \cdot u^2(p) \\ & + \left(\frac{\partial \mathrm{cHCQ}}{\partial V_{flask}}\right)^2 \cdot u^2(V_{flask}) + \left(\frac{\partial \mathrm{cHCQ}}{\partial V_{p,stock}}\right)^2 \cdot u^2(V_{p,stock}) \\ & + \left(\frac{\partial \mathrm{cHCQ}}{\partial V_{p,water}}\right)^2 \cdot u^2(V_{p,water}) + \left(\frac{\partial \mathrm{cHCQ}}{\partial V_{p,ws}}\right)^2 \cdot u^2(V_{p,ws}) \\ & + \left(\frac{\partial \mathrm{cHCQ}}{\partial V_{p,blood}}\right)^2 \cdot u^2(V_{p,blood}) \end{aligned} \tag{15}$$

Performing partial derivatives using Eq. (1), substituting them in Eq. (14), and rearranging terms, Eq. (15) becomes:

$$u_c(\mathrm{cHCQ}) = \mathrm{cHCQ} \cdot \sqrt{\begin{aligned} & \left[\frac{u(m)}{m}\right]^2 + \left[\frac{u(p)}{p}\right]^2 + \left[\frac{u(V_{flask})}{V_{flask}}\right]^2 \\ & + \left(\frac{V_{p,water}}{V_{p,stock}+V_{p,water}}\right)^2 \cdot \left[\frac{u(V_{p,stock})}{V_{p,stock}}\right]^2 + \left[\frac{u(V_{p,water})}{V_{p,stock}+V_{p,water}}\right]^2 \\ & + \left(\frac{V_{p,blood}}{V_{p,ws}+V_{p,blood}}\right)^2 \cdot \left[\frac{u(V_{p,ws})}{V_{p,ws}}\right]^2 + \left[\frac{u(V_{p,blood})}{V_{p,ws}+V_{p,blood}}\right]^2 \end{aligned}}$$

or, in its relative form:

$$u_{rel,c}(\mathrm{cHCQ}) = \sqrt{\begin{aligned} & u_{rel}^2(m) + u_{rel}^2(p) + u_{rel}^2(V_{flask}) \\ & + \left(\frac{V_{p,water}}{V_{p,stock}+V_{p,water}}\right)^2 \cdot \left[u_{rel}^2(V_{p,stock}) + u_{rel}^2(V_{p,water})\right] \\ & + \left(\frac{V_{p,blood}}{V_{p,ws}+V_{p,blood}}\right)^2 \cdot \left[u_{rel}^2(V_{p,ws}) + u_{rel}^2(V_{p,blood})\right] \end{aligned}}$$

The steps followed to obtain the final expressions of Eq. (15) from Eq. (1) and Eq. (14) are included in the Supplementary material.

2.4.5. Calculation of the expanded uncertainty

The expanded uncertainty, $U(\text{cal})$, was calculated by multiplying the $u_c(\text{cal})$ by an adequate coverage factor. Under typical clinical laboratory working conditions, it is acceptable to use a *k*-value of 2 [14]:

$$U(\text{cal}) = 2 \cdot u_c(\text{cal}) \quad (16)$$

2.4.6. Expression of measurement uncertainty

The MU expression of each end-user calibration material value (cHCQ) was expressed as [14]:

$$\text{cHCQ} \pm U(\text{cal})$$

Also, a coverage interval could be used to express an end-user calibration material result:

$$[\text{cHCQ} - U(\text{cal}); \text{cHCQ} + U(\text{cal})]$$

3. Results

Table 2 shows the value assigned to each end-user calibration material, the MU budget, the combined and expanded uncertainties, as well as the coverage intervals. Table 3 depicts the same information as Table 2 but shows relative uncertainties (in %) instead of absolute values. In these conditions, the source of uncertainty with the greatest contribution was that related to the HCQ RM mass obtained via balance, while the uncertainty associated with HCQ RM purity was the least important.

4. Discussion

At present, it is widely accepted that MU information may help in the interpretation of measurement values provided by a clinical laboratory. Also, MU could have an impact on clinicians' decision-making, especially when values are compared with biological reference intervals, therapeutic intervals or clinical decision values [14–17].

The so-called *top-down* approach is particularly well suited to estimation of the MU in clinical laboratories. Uncertainties associated with long-term intermediate precision, bias, and values assigned to end-user calibration materials are the most significant contributions to consider. The first two sources of uncertainty are estimated from measurement procedure validation or verification data or intra/inter-laboratory quality control (internal or external) data. By contrast, for the $u(\text{cal})$, data provided by manufacturers are usually used [14–17]. Unfortunately, clinical laboratories consider that the $u(\text{cal})$ is negligible when they produce end-user calibration materials. Moreover, if laboratories do estimate this MU, they often calculate it by simple aggregation of the standard uncertainties coming from different sources without regard for the actual *measurement equation*. The estimation of $u(\text{cal})$ should be achieved by an evaluation of the appropriate measurement equation which describes the interaction of all the relevant components which combine to provide the actual calibration material value. This measurement equation must be included when the measurand is defined. The uncertainty associated with this calibration material value can then be calculated by application of the GUM *law for the propagation of uncertainty*, with appropriate differentiation and determination of the relevant *sensitivity coefficients* [13].

To our knowledge, there are no published studies related to the estimation of uncertainty associated with the values assigned to end-user calibration materials prepared *in-house* by a clinical laboratory. Thus, we aimed to estimate the $u(\text{cal})$ for the mass concentration of HCQ in blood-hemolysate-based matrix to exemplify how clinical laboratories could calculate it.

In general terms, the most important uncertainty contribution in the $u(\text{cal})$ was the one associated with the RM mass obtained via balance because of the inherent error in weighing a small amount of this material. This uncertainty source could be reduced by weighing a greater amount of RM, a measure that is not always possible due to the high cost of these materials. Also, we could use a ready-to-use liquid RM, if this is available. Currently, different manufacturers exist that supply this RM (LGC standards, Sigma-Aldrich, Cerilliant, among others) along with its uncertainty, and that could be used to prepare the stock solution.

Conversely, in our study, the smallest contribution to the uncertainty budget was that associated with the RM's purity, and it can be considered negligible compared to the other sources of uncertainty. However, if a laboratory uses a RM that presents a higher uncertainty related to its purity, this uncertainty could have a non-negligible contribution.

Regarding the $u(V_{\text{p.stock}})$ and the $u(V_{\text{p.water}})$, their contributions to the uncertainty budget vary depending on the volume pipetted. The relative

Table 2
Measurement uncertainty budget (in absolute values) for hydroxychloroquine end-user calibration material values.

Level	Calibrator assigned value (μg/L)	HCQ RM mass-weighed	HCQ RM Purity	Volumetric flask volume	Pipetted volumes				u_c(cHCQ) (μg/L)	U(cHCQ) (μg/L)	Coverage interval (μg/L)
		$u(m)$ (mg)	$u(p)$ (g/g)	$u(V_{\text{flask}})$ (mL)	$u(V_{\text{p,stock}})$ (μL)	$u(V_{\text{p,water}})$ (μL)	$u(V_{\text{p,ws}})$ (μL)	$u(V_{\text{p,blood}})$ (μL)			
1	49.9	0.1000	$1.411 \cdot 10^{-4}$	0.0209	0.0701	2.0000	0.1000	0.6000	1.3361	2.7	[47.3; 52.6]
2	99.9	0.1000	$1.411 \cdot 10^{-4}$	0.0209	0.1001	2.0000	0.1000	0.6000	2.4855	5.0	[94.9; 104.8]
3	199.7	0.1000	$1.411 \cdot 10^{-4}$	0.0209	0.2500	2.0000	0.1000	0.6000	5.1752	10.4	[189.4; 210.1]
4	399.5	0.1000	$1.411 \cdot 10^{-4}$	0.0209	0.4000	2.0000	0.1000	0.6000	9.8936	19.8	[379.7; 419.3]
5	599.2	0.1000	$1.411 \cdot 10^{-4}$	0.0209	0.4000	2.0000	0.1000	0.6000	14.1861	28.4	[570.8; 627.6]
6	998.7	0.1000	$1.411 \cdot 10^{-4}$	0.0209	0.6000	2.0000	0.1000	0.6000	23.4292	46.9	[951.8; 1046]
7	1498	0.1000	$1.411 \cdot 10^{-4}$	0.0209	1.1500	2.0000	0.1000	0.6000	35.5661	71	[1427; 1569]
8	1997	0.1000	$1.411 \cdot 10^{-4}$	0.0209	1.1500	2.0000	0.1000	0.6000	46.5166	93	[1904; 2090]

HCQ; hydroxycloroquine; RM, reference material; $u(m)$, uncertainty associated with the HCQ reference material mass-weighed into the balance; $u(p)$, uncertainty related to the purity of the HCQ reference material; $u(V_{\text{flask}})$, uncertainty associated with the volumetric flask volume used to prepare the HCQ stock solution; $u(V_{\text{p.stock}})$, uncertainty related to the volume of stock solution pipetted to prepare the corresponding HCQ working standard solution; $u(V_{\text{p.water}})$, uncertainty associated with the volume of LC/MS-grade water pipetted to prepare the corresponding HCQ working standard solution; $u(V_{\text{p.ws}})$, uncertainty associated with the volume of the corresponding working standard solution pipetted to prepare the appropriate HCQ calibration material; $u(V_{\text{p.blood}})$; uncertainty related to the volume of the corresponding drug-free hemolysate solution pipetted to prepare the appropriate HCQ calibration material; u_c(cHCQ), combined standard uncertainty related to the mass concentration of hydroxychloroquine in blood based-matrix calibration materials;U(cHCQ), expanded uncertainty related to the mass concentration of hydroxychloroquine in blood based-matrix calibration materials.

Table 3
Measurement uncertainty budget (in relative values) for hydroxychloroquine end-user calibration material values.

Level	Calibrator assigned value (μg/L)	HCQ RM mass-weighed	HCQ RM Purity	Volumetric flask volume	Pipetted volumes				$u_{c,rel}$(cHCQ) (%)	U_{rel}(cHCQ) (%)
		$u_{rel}(m)$ (%)	$u_{rel}(p)$(%)	$u_{rel}(V_{flask})$(%)	$u_{rel}(V_{p,stock})$(%)	$u_{rel}(V_{p,water})$(%)	$u_{rel}(V_{p,ws})$(%)	$u_{rel}(V_{p,blood})$(%)		
1	49.9	2.000	0.0141	0.0417	1.4026	0.2000	1.0000	0.6666	2.6757	5.4
2	99.9	2.000	0.0141	0.0417	1.0009	0.2020	1.0000	0.6666	2.4887	5.0
3	199.7	2.000	0.0141	0.0417	1.2502	0.2041	1.0000	0.6666	2.5909	5.2
4	399.5	2.000	0.0141	0.0417	1.0001	0.2083	1.0000	0.6666	2.4766	5.0
5	599.2	2.000	0.0141	0.0417	0.6667	0.2128	1.0000	0.6666	2.3674	4.7
6	998.7	2.000	0.0141	0.0417	0.6000	0.2222	1.0000	0.6666	2.3459	4.7
7	1498	2.000	0.0141	0.0417	0.7667	0.2353	1.0000	0.6666	2.3741	5
8	1997	2.000	0.0141	0.0417	0.5750	0.2500	1.0000	0.6666	2.3288	5

HCQ; hydroxycloroquine; RM, reference material; $u_{rel}(m)$, relative uncertainty associated with the HCQ reference material mass-weighed into the balance; $u_{rel}(p)$, relative uncertainty related to the purity of the HCQ reference material; $u_{rel}(V_{flask})$, relative uncertainty associated with the volumetric flask volume used to prepare the HCQ stock solution; $u_{rel}(V_{p.stock})$, relative uncertainty related to the volume of stock solution pipetted to prepare the corresponding HCQ working standard solution; $u_{rel}(V_{p.water})$, relative uncertainty associated with the volume of LC/MS-grade water pipetted to prepare the corresponding HCQ working standard solution; $u_{rel}(V_{p.ws})$, relative uncertainty associated with the volume of the corresponding working standard solution pipetted to prepare the appropriate HCQ calibration material; $u_{rel}(V_{p.blood})$; relative uncertainty related to the volume of the corresponding drug-free hemolysate solution pipetted to prepare the appropriate HCQ calibration material; $u_{c,rel}$(cHCQ), relative combined standard uncertainty related to the mass concentration of hydroxychloroquine in blood based-matrix calibration materials;U_{rel}(cHCQ), relative expanded uncertainty related to the mass concentration of hydroxychloroquine in blood based-matrix calibration materials.

$u(V_{p.stock})$ values will decrease as the calibrator values increase, because more volume is pipetted and less associated error exists with increasing volume, but for the $u(V_{p.water})$, the opposite will occur. In addition, from the combined measurement uncertainty equation, it can be deduced that the contributions of these uncertainties will be less than they really are because both uncertainties are always multiplied by the factor $\frac{V_{p,water}}{V_{p,stock}+V_{p,water}}$ that will provide values less than 1. Moreover, this factor also decreases as the calibrator values increase, given that the water volume pipetted to prepare the working standard solution increases with the increasing value of the calibration material.

For the $u(V_{p.ws})$ and the $u(V_{p.blood})$, their contributions will be constant for all calibration values given that the volumes pipetted are always the same. Besides, these uncertainties will also be lower than they really are for the same reason mentioned above.

Although the u(cal) evaluation method demonstrated is simple and would be easy to implement in clinical laboratories, we have to indicate that the estimation procedure presented here will probably produce an underestimation of the u(cal) because we did not consider all possible sources of uncertainty. For example, we did not take into account the lack of homogeneity of calibration materials, the within-batch variability of the preparation of the calibration materials, the influence of the density of the solvents, the loss of stability of the end-user calibration materials, the adhesion of HCQ to the different glass and plastic materials used (non-specific binding studies), as well as the effect of the evaporation of the solvents. Despite this, to simplify the u(cal) calculation and to facilitate the task and comprehension of clinical laboratories, we have only considered those sources of uncertainty and studies that surely any laboratory would have taken into account when they would prepare their calibration materials.

5. Conclusions

In summary, this study shows how the u(cal) can be estimated for the mass concentration of HCQ in blood-hemolysate-based matrix. The procedure presented could be put into practice for other pharmacological quantities measured by *in-house* HPLC or HPLC-MS/MS procedures commonly used in clinical laboratories.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.clinbiochem.2021.01.005.

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