Available online at www.sciencedirect.com
ScienceDirect
Journal of Pharmaceutical and Biomedical Analysis 42 (2006) 56–63
JOURNAL OF PHARMACEUTICAL AND BIOMEDICAL ANALYSIS
www.elsevier.com/locate/jpba

Uncertainty evaluation in the chloroquine phosphate potentiometric titration: Application of three different approaches

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Received 18 October 2005; received in revised form 22 May 2006; accepted 30 May 2006
Available online 25 July 2006

Abstract

A measurement result cannot be properly interpreted if not accompanied by its uncertainty. Several methods to estimate uncertainty have been developed. From those methods three in particular were chosen in this work to estimate the uncertainty of the Eu. Ph. chloroquine phosphate assay, a potentiometric titration commonly used in medicinal control laboratories. The famous error-budget approach (also called bottom-up or step-by-step) described by the ISO Guide to the expression of Uncertainty in Measurement (GUM) was the first method chosen. It is based on the combination of uncertainty contributions that have to be directly derived from the measurement process.

The second method employed was the Analytical Method Committee top-down which estimates uncertainty through reproducibility obtained during inter-laboratory studies. Data for its application were collected in a proficiency testing study carried out by over 50 laboratories throughout Europe.

The last method chosen was the one proposed by Barwick and Ellison. It uses a combination of precision, trueness and ruggedness data to estimate uncertainty. These data were collected from a validation process specifically designed for uncertainty estimation.

All the three approaches presented a distinctive set of advantages and drawbacks in their implementation. An expanded uncertainty of about 1% was assessed for the assay investigated.

Keywords: Measurement uncertainty; Error-budget; Bottom-up; Top-down; Error-propagation; Barwick and Ellison; Titration assay

1. Introduction

According to the "International Vocabulary of Basic and General Terms in Metrology" [1], uncertainty of measurement is a parameter associated with the result of a measurement, that characterises the dispersion of the values that could reasonably be attributed to the measurand.

Nowadays it is widely recognised that without knowledge of the measurement uncertainty, the statement of an analytical result cannot be considered complete.

Uncertainty knowledge is also fundamental in assessing compliance with limit values specified in regulations [2]. The ISO/IEC 17025 itself [3] recognizes the vital role of uncertainty of measurement, stating that testing and calibration laboratories shall have and apply procedures for its estimation. Thus, in the last few years a great effort has been put into setting out methods capable of quantifying and expressing uncertainty and nowadays several different approaches are at chemists' disposal.

Thus the aim of this work was to implement some of the above approaches to evaluate the uncertainty in the *chloroquine phosphate* assay. Chloroquine phosphate is an antimalaric drug which assay is of very common use in medicinal control laboratories. It is based on a potentiometric titration reported in the *European Pharmacopoeia*. Of the various general methods for uncertainty estimation that are reported in literature and guidlines, three in particular were chosen.

The first one was originally developed by metrologists and physicists and proposed in the ISO "Guide to the expression of Uncertainty in Measurement" (GUM) [4], and only recently adopted by EURACHEM for analytical chemistry [5]. It evaluates the overall uncertainty by identifying, estimating and combining all the sources of uncertainty associated with the measurement process. Because of the way the overall uncertainty is assessed it has been defined as *error-budget* or *bottom-up*

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doi:10.1016/j.jpba.2006.05.026

approach [4–6]. It has been the object of a deep debate in the analytical community because on one hand it has the evident advantage of permitting to identify the significant sources of uncertainty in the measurement procedure, making it easy to understand which parts need to be treated with care or which need to be improved [6]. On the other hand, as often remarked by Horwitz [16,17] it is complicated by practical problems like overlooking important variables, double counting others and the presence of unknown interactions and interferences. Visser, collecting the practical experiences of many accredited testing laboratories, was able to demonstrated that, when complex analytical methods with interfering matrixes or sampling steps are involved, the ISO GUM *error-budget* approach often produces uncertainty estimates not comparable with those derived from validation data or inter-laboratory studies [18,19]. Similarly Hund et al. comparing different approaches to estimate uncertainty for a complex analytical method such as liquid chromatography, came to the conclusion that the GUM uncertainty estimates are clearly smaller then the estimates from the other approaches [20]. Hund et al. [15] stressed that the *error-budget* method as described by the ISO GUM is suitable for physical measurements, while it proves to be hardly applicable for complex analytical methods because of the difficulty in constructing the error-budget and in avoiding overlooking of error sources. Hence they strongly recommended the use of validation and quality assurance data for uncertainty estimation. Other authors such as Furman et al. [21] have arrived independently to similar conclusions. EURACHEM itself, answering to the rising requests of the analytical community, has finally introduced a mention to validation data and inter-laboratory study in its last version [22]. Nowadays although with some commendable exceptions (e.g. [23]), when complex analytical methods are involved, the trend goes toward using other more holistic approaches [24–29]. Even if the *chloroquine* assay considered in this study is a simple primary method and Hund et al. said that the GUM approach is probably suitable for primary analytical methods [15], as a matter of fact no effort to date have been put into verifying this hypothesis. Thus, in this work it was deemed sensible to adopt also some more holistic approaches to evaluate the uncertainty of interest.

Hence a second approach was considered: the commonly named *top-down* introduced by Wernimont [7]. This approach was developed by the Analytical Methods Committee [8] and recently recommended by ISO in the ISO/TS 21748 [9]. It uses data obtained by inter-laboratory studies performed in accordance with the harmonized IUPAC/AOAC protocol or ISO 5725 [10] to asses uncertainty.

The third method chosen is based on the possibility to estimate uncertainty from the information gathered during method validation and other quality assurance procedures. This option can be conceptually regarded as a mix of the first two methods as it is indeed similar to the *top-down* because of its holistic character but is also consistent with the *bottom-up* since the error sources are identified, quantified and combined [11]. A great effort was given to the development of this approach by Maroto et al. [11,12], Barwick and Ellison [13,14] and Hund et al. who in particular clarified the relationship between validation and uncertainty, giving different operational definitions of uncertainty depending on the situation under which the analyst is validating [15].

In the present study the Barwick and Ellison approach was chosen.

Finally also the possibility to use the well-known *Horwitz equation* [30], that permits to theoretically predict the reproducibility of an analytical method, was taken into account.

The uncertainty assessed in this work dealt only with the measurement process, uncertainty due to sampling was not considered.

2. Experimental

2.1. Analytical methods

As indicated in the *European Pharmacopoeia* [31] the chloroquine phosphate assay consists of a non-aqueous acid–base titration with potentiometric end-point detection.

The titrator used was a Metrohm model 726 with an automatic burette of 10 ml capacity.

The balance used was a Gibertini model E50S with a last digit of 0.1 mg.

All the instruments were properly qualified and their performance was checked as prescribed by the authors' quality assurance system.

All calculations were performed with the Microsoft Excel Software of the Office XP Package.

The analysis was conducted on the active substance itself and not on the medicinal product. Perchloric acid ($HClO_4$), the titre of which was determined against potassium hydrogen phthalate (KHP) was used as the titrating agent and acetic acid was used as solvent. The result was expressed as a percentage by mass.

A single analysis consisted of the following steps (each value reported in parentheses is a fixed constraint of the method [31,32] and no discretion was left to the analyst):

(1) $HClO_4$ titre determination:
   (a) weighing KHP (about 100 mg);
   (b) KHP titration with $HClO_4$ (about 0.1 M);
   (c) $HClO_4$ titre determination using the formula:

$$C_{HClO_4} = \frac{(m_{KHP}/M_{KHP})}{V'_{HClO_4}} \quad (1)$$

   where C_{HClO_4} is the concentration of the $HClO_4$ solution [mol l^{-1}], m_{KHP} mass of KHP taken [mg], V'_{HClO4} volume of $HClO_4$ solution used to titrate KHP [ml] and M_{KHP} is the molar mass of KHP [g mol^{-1}].
   (d) the a–c steps were repeated three times and a mean value ($\bar{C}_{HClO_4}$) was calculated for the $HClO_4$ titre.

(2) Chloroquine phosphate titre determination:
   (a) weighing chloroquine (about 200 mg);
   (b) chloroquine titration with $HClO_4$ (about 0.1 M);

(c) chloroquine titre calculation using the formula:

$$\text{Titre}\% = \frac{V''_{\text{HClO}_4} 25.79 C_{\text{HClO}_4}}{m_{\text{chloroquine}} 0.1} 100\% \quad (2)$$

where C_{HClO_4} is the concentration of the $HClO_4$ solution [mol l^{-1}], V''_{HClO_4} volume of $HClO_4$ solution used to titrate chloroquine phosphate [ml], $m_{\text{chloroquine}}$ mass of chloroquine phosphate taken [mg], 25.79 milligrams of chloroquine titrated by 1 ml of 0.1 M $HClO_4$ and 0.1 is the concentration [mol l^{-1}] of the ideal $HClO_4$ solution that titrates 25.79 mg of chloroquine.

(d) the a–c steps were repeated three times and a mean value ($\overline{\text{Titre}}\%$) was calculated for the chloroquine titre.

To accept the titration procedure and verify whether it was properly transferred to authors' laboratory, its performance was checked as stated by the "volumetric titration" chapter of the *European Pharmacopoeia* technical guide [32]. All the criteria set by the guide were fulfilled.

2.2. Materials

Chloroquine phosphate was purchased from Fluka BioChemica (purity >96%). $HClO_4$ was purchased from Sigma–Aldrich (declared concentration: 0.099N). KHP standard for volumetric analysis were purchased from Sigma–Aldrich and Carlo Erba. Reagent grade glacial acetic acid was purchased from Rudi Pont (purity >99.8%).

2.3. Uncertainty estimation

2.3.1. ISO GUM error-budget approach

To apply the *error-budget* approach the procedure fully described in the ISO GUM [4] is to be followed. This procedure comprises the following steps:

(a) uncertainty sources are identified (e.g. instrument effects, random effects, reagent purity, uncertainty of weights and volumetric equipments, etc.).
(b) Each source is quantified. One should make an approximate assessment of size of the contribution from each source.
(c) Each quantified source is expressed as a relative standard deviation. Each of these separate contributions is called an uncertainty component or a standard uncertainty.
(d) The various uncertainty components are combined by the classical error-propagation algorithm and a combined uncertainty is obtained.
(e) The final result is reported as an expanded uncertainty. This is obtained by multiplying the combined uncertainty by a coverage factor K. In practice a value of $K = 2$ is suitable for most circumstances. When the distributions of the various uncertainty components are deemed normal, a value of $K = 2$ roughly corresponds to a 95% confidence level (the best coverage factor could be obtained as two tailed Student's t-test at a 95% confidence level for a number of degrees of freedom determined by the Welch–Satterthwaite formula. Unfortunately this procedure is far too laborious in most of the cases. Alternatively the Williams procedure can be employed [33]).

To determine the chloroquine titre uncertainty the procedure indicated in [22, examples A2 and A3] for acid–base titrations and the scheme proposed by Anglov et al. [34] served as guidance. Hence just a brief description of the various steps will be given in this paper. Interested readers are welcome to write the corresponding author and ask for the detailed version of the text (which includes all the formulas employed and calculations performed). The requested material will be promptly supplied by e-mail.

The uncertainty sources were identified using a cause–effect diagram constructed in accordance with the procedure indicated in [22] (the diagram is depicted in Fig. 1) and rearranging the formulas (1) and (2) so that each of their terms represents an uncertainty source:

$$\text{Titre}\% = \frac{V''_{\text{HClO}_4} 25.79 C_{\text{HClO}_4}}{m_{\text{chloroquine}} 0.1} 100\% \quad (3)$$

$$C_{\text{HClO}_4} = \frac{(m_{\text{KHP}}/M_{\text{KHP}})}{V'_{\text{HClO}_4}} P(\text{KHP}) \quad (4)$$

where P(KHP) is purity of KHP given as mass fraction; the other variables have already been defined in Section 2.1.

Each source was then quantified and expressed as a relative standard deviation. Here follows a list and a brief description of the uncertainty contribution considered.

- *Uncertainty associated with KHP and chloroquine mass*

  Repeatability, resolution, eccentricity and linearity were considered.

  Weighing repeatability was taken from the balance calibration certificate. Balance digital resolution and eccentricity resulted negligible. Linearity is defined in [22] as the maximum difference between the actual mass on the pan and the reading of the scale. As for repeatability its value was reported on the balance calibration certificate. All the contributions were counted twice, once for the tare and once for the gross weight.

  One can observe that the balance nonlinearity should only have a minor effect when the total mass and vessel mass differ for less then 1 g. However, it was decided to treat the linearity contribution in the same way as reported in the Eurachem examples [22] without further investigations.

- *Uncertainty associated with $HClO_4$ volumes used to titrate KHP and chloroquine*

  Two contributions to this uncertainty were considered: one from calibration and one from temperature's effect. For the first one both burette accuracy limits and repeatability were assessed. For the second one a temperature variation during the experiment of ±3 °C was derived from the quality assurance temperature control charts. Since the titrant ($HClO_4$) was dissolved in acetic acid the acetic acid's volume expansion coefficient [35] was used. It should be noticed that no correction for the temperature effect is included in the *European*

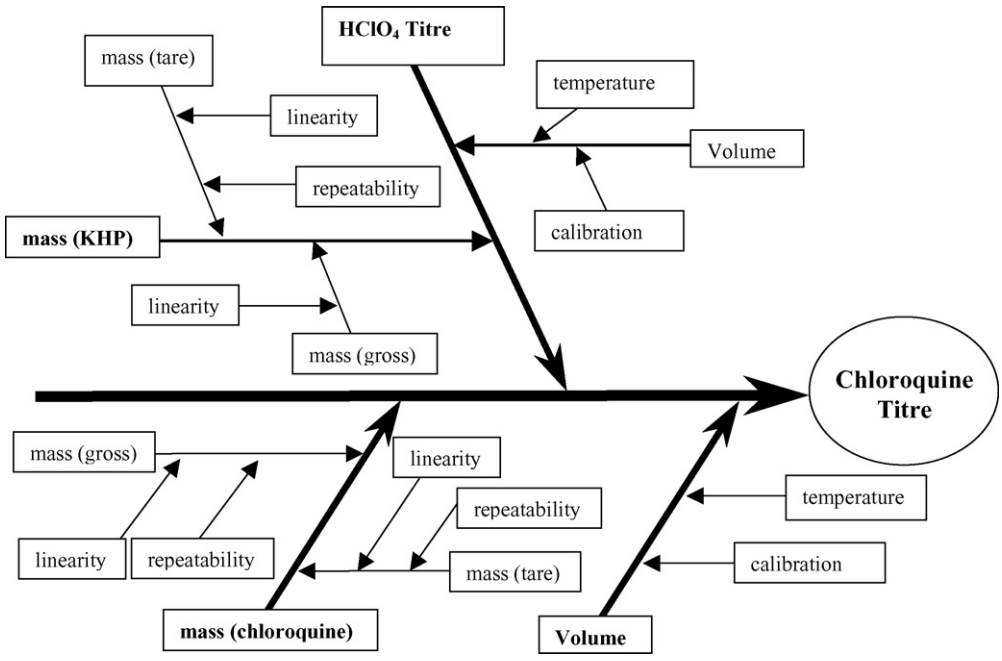

Fig. 1. Cause–effect diagram for the chloroquine phosphate titre determination. Each source contributing to the overall uncertainty is depicted.

Pharmacopoeia method because the operator is supposed to determine the chloroquine titre immediately after the titrant one. All the same a laboratory temperature variation of several degrees is reasonably expected even in short periods of time when no temperature control is exerted. Since this variation is not accounted for in the analytical method, here it has been considered as an uncertainty source.

- *Uncertainty associated with molar mass of KHP and chloroquine phosphate*

  Their contributions were calculated but resulted negligible.

- *Uncertainty associated with KHP purity*

  Purity was indicated in the supplier certificate.

All the various contributions just mentioned were then combined in two steps: in the first one the combined standard uncertainty for $HClO_4$ titre was determined

$$u_{\text{titrant}} = \sqrt{(u^{\text{TOT}}_{m(\text{KHP})})^2 + (u^{\text{TOT}}_{\text{volume}})^2 + (u_{\text{Purity(KHP)}})^2} \quad (5)$$

in the second one the combined standard uncertainty for chloroquine titre was calculated with Eq. (5) considered as one of the contributions:

$$u_{\text{chloroquine}} = \overline{\text{Titre}}\% \sqrt{(u^{\text{TOT}}_{m(\text{chloroquine})})^2 + (u^{\text{TOT}}_{\text{volume}})^2 + (u_{\text{titrant}})^2} \quad (6)$$

As an alternative the combined uncertainty was determined including the experiment repeatability taken from a previous validation, as suggested in [22]. Repeatability of the volume delivered by the burette and repeatability of the weighing operation were considered included in this general repeatability term and thus neglected in the calculation.

This procedure of course cannot be considered to accord strictly to the ISO GUM approach, so it was evaluated just as another possible way of estimating uncertainty together with those described in the following paragraphs.

2.3.2. The Analytical Methods Committee top-down approach: uncertainty estimation from data collected in inter-laboratory studies

This approach estimates the uncertainty of measurement from the reproducibility standard deviation obtained by collaborative trials. In this approach the laboratory is seen from a higher level (i.e. as a member of the population of laboratories) so systematic and random errors within individual laboratories become random errors when they are considered from this "higher level" [12]. The method is simple and straightforward. It is commonly considered utterly reliable since the reproducibility calculated from an inter-laboratory study undoubtedly covers the widest range of possible uncertainty sources. The reproducibility standard deviation, in fact, accounts for the whole analytical process, from reception and storage of samples to the experimental work in the laboratory.

Reproducibility data were taken from an European Directorate on the Quality of Medicines (EDQM) collaborative trial (the so-called "Proficiency Testing Study" or PTS) conducted on 58 laboratories [36]. Outliers were indicated using three test statistics [10]: Cochran's test for outlying variances, Grubbs' single test for outlying means and Grubbs' paired test for outlying means, applied in this order. If a laboratory was excluded as an outlier, the cycle was repeated from Cochrane's test until no outliers remained. Six laboratories were deemed outliers and excluded from the uncertainty computation. Following the model proposed in [10], reproducibility standard deviation was calculated combining within-laboratory and between-laboratory

standard deviations:

$$s_R = \sqrt{s_L^2 + s_r^2} \quad (7)$$

where s_L is the between-laboratory standard deviation and was calculated over the 52 mean values of the chloroquine titre collected by the EDQM (the 58 participating laboratories minus the 6 outliers); s_r is the within-laboratory standard deviation. It is the arithmetic mean of the repeatability standard deviations of all those laboratories taking part in the trial which remain after outliers have been excluded.

The expanded uncertainty was then obtained multiplying the reproducibility standard deviation by 2, the coverage factor for a 95% confidence level in a normal distribution hypothesis:

$$U_{\text{top-down}} = 2s_R \frac{\overline{\text{Titre}}\%}{\overline{\text{Titre}}\%_{\text{PTS}}} \quad (8)$$

where $\overline{\text{Titre}}\%$ is the same of (6) and has already been defined in Section 2.1, while $\overline{\text{Titre}}\%_{\text{PTS}}$ is the mean titre determined during the PTS.

The possibility to use the Horwitz function [30] to evaluate reproducibility was taken into account but promptly discarded because of the intrinsic high precision of the analytical method considered in this work. The non-aqueous acid–base titration with potentiometric end-point detection is indeed a too precise analytical method to follow the Horwitz rule. This rule was actually derived from a class of collaborative trials regarding much less precise methods such as the chromatographic ones [37,38]. Thus, it was judged wiser to discard the results suggested by the Horwitz equation to avoid a major overestimation of uncertainty.

2.3.3. Barwick and Ellison approach [13,14,39]: uncertainty estimation from a combination of validation data

The Barwick and Ellison (B&E) approach permits to estimate uncertainty trough a proper combination of validation data. The procedure is very well described in the protocol reported in [39]. The main experimental studies are for the evaluation of precision and trueness. These should be planned so as to cover as many sources of uncertainty identified for the method as possible. Any remaining source is to be considered separately. Alternatively they can be accounted for simultaneously by carrying on carefully planned ruggedness study. This second possibility was chosen in this work. Data collected from precision, trueness and ruggedness experiments are then to be combined as standard uncertainties in the same way stated by the ISO GUM and the Eurachem Guide.

Therefore, the analytical method for chloroquine titration was completely validated in accordance to the prescriptions of [39].

Precision studies were divided into intra and inter-day, respectively.

The inter-day precision (repeatability) was assessed by ten independent measurements of the chloroquine titre. Each one comprised three determination of the perchloric acid titre and three determination of the chloroquine titre as described in Section 2.1.

A mean value and a standard deviation were calculated. The relative standard deviation of the sample was used as relative standard uncertainty in the uncertainty budget evaluation.

The inter-day precision was determined measuring the chloroquine titre over four different days. Each day five different determination of the titre were performed and the outliers were discarded. A sample standard deviation was calculated and used as a standard uncertainty.

For trueness studies a certified reference material (CRM) was available so trueness was estimated in terms of overall recovery, i.e. the ratio of the observed value to the expected value. Three determinations were performed on the CRM and a recovery value was calculated using the equations from "trueness study" section of [39]. B&E recommend to perform at least 10 determinations but unfortunately not enough certified reference substance was at author disposal. As suggested in [39] a proper Student's *t*-test value was applied to account for the fewer replicates. This of course determined a large trueness contribution to the overall uncertainty.

Finally ruggedness experiments were planned and performed to test the effect of the temperature change during the analysis and the effect of changing the primary titration standard (KHP). The Plackett–Burman experimental design was used as a guidance for experimental planning [40]. Each parameter was investigated at two levels as follows:

(1) Temperature: 20 °C was chosen as the initial level and 25 °C as the alternative one. The temperature was changed between the titrant and chloroquine titrations of the same assay.
(2) Primary standard: A sample provided by the usual supplier and a sample from a different one were used.

Four experiments were performed following the scheme reported in [39,40].

Through ruggedness studies a temperature sensitivity coefficient of 0.077% °C^{-1} was assessed, meaning that a 0.077% of variation in chloroquine titre value is observed for every degree of temperature variation during the experiment.

Results collected from these ruggedness studies demonstrated that changing the KHP supplier does not affect the method performance, while the temperature has a significant effect. So according to B&E the two contributions to the final uncertainty were calculated differently.

Eventually all the contributions mentioned were combined using the error-propagation algorithm and multiplied by the mean titre mentioned in Section 2.1:

$$u_{\text{combined}} = \overline{\text{Titre}}\% \sqrt{(u_{\text{repeatability}})^2 + (u_{\text{inter-day}})^2 + (u_{\text{trueness}})^2 + (u_{\text{ruggedness}}^{\text{KHP}})^2 + (u_{\text{ruggedness}}^{T})^2} \quad (9)$$

Table 1
Uncertainty sources considered in the ISO GUM *error-budget* approach

Symbol	Description	Mean value	Standard uncertainty	Relative standard uncertainty
m_{KHP}	Mass of KHP	90.8 mg	0.3020 mg	$u^{TOT}_{m(KHP)} = 0.003326$
$m_{chloroquine}$	Mass of chloroquine phosphate	178.3 mg	0.3020 mg	$u^{TOT}_{m(chloroquine)} = 0.001694$
V'_{HClO_4}	Volume of $HClO_4$ solution used to titrate KHP	4.489 ml	0.01054 ml	$u'^{TOT}_{volume} = 0.002347$
V''_{HClO_4}	Volume of $HClO_4$ solution used to titrate chloroquine phosphate	6.797 ml	0.01416 ml	$u''^{TOT}_{volume} = 0.002084$
P(KHP)	Purity of KHP given as mass fraction	1.0	0.0002887	$u_{Purity(KHP)} = 0.0002887$
C_{HClO_4}	Concentration of the $HClO_4$ solution	0.09903 mol l^{-1}	0.0004042 mol l^{-1}	$u_{titrant} = 0.004081$
Titre%	Titre of the chloroquine phosphate	97.35%	0.4756%	0.004885

Explanation of the symbols and results of the calculations.

The final uncertainty was expressed as an expanded uncertainty. In order to obtain the expanded uncertainty, the combined uncertainty obtained in (9) was multiplied by 2, the coverage factor for a 95% confidence level in a normal distribution hypothesis.

3. Results

3.1. ISO GUM error-budget approach

Results of the calculations are summarized in Table 1 in which the calculated values for the parameters of formulas (3) and (4) are reported together with their uncertainty contributions.

The final result was expressed as an expanded uncertainty, multiplying the combined relative standard uncertainty of the titre (6) by 2, the coverage factor for a 95% confidence level in a normal distribution hypothesis. Thus, an expanded uncertainty of 0.95% was obtained.

In Fig. 2 the various contributions to the overall uncertainty are depicted.

A value of 1.05% for expanded uncertainty was obtained when a validation repeatability term was included in the calculation (thus neglecting repeatabilities associated with volume delivery and weight operation) as described in the last part of Section 2.3.1.

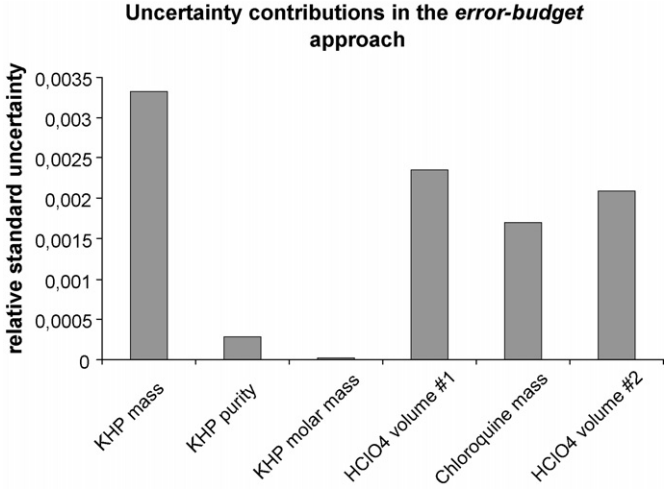

Fig. 2. The relative uncertainties contributing to the combined uncertainty in the *ISO GUM error-budget* approach.

Table 2
All the formulas used to calculate uncertainty by the *top-down* approach and the corresponding results

Symbol	Description	Uncertainty formulas and results
s_R	Reproducibility standard deviation	$s_R = \sqrt{s_L^2 + s_r^2} = 0.5183\%$
s_L	Between-laboratory standard deviation	$s_L = 0.4924\%$
s_r	Within-laboratory standard deviation.	$s_r = 0.1620\%$
$U_{top\text{-}down}$	Expanded uncertainty	$U_{top\text{-}down} = 2s_R \frac{\overline{Titre}\%}{\overline{Titre}\%_{PTS}} = 1.032\%$

For brevity's sake only four significant figures were reported. To avoid truncation errors a spreadsheet was used to perform calculations.

3.2. Analytical Method Committee top-down approach

From the application of the procedure indicated in Section 2.3.2 an expanded uncertainty of 1.03% was obtained. The within-laboratory and between-laboratory standard deviation values together with the formula used for combining them are reported in Table 2.

3.3. Barwick and Ellison approach

The procedure described in Section 2.3.3 brought an expanded uncertainty of 1.02%.

In Fig. 3 the various contributions to the overall uncertainty are depicted.

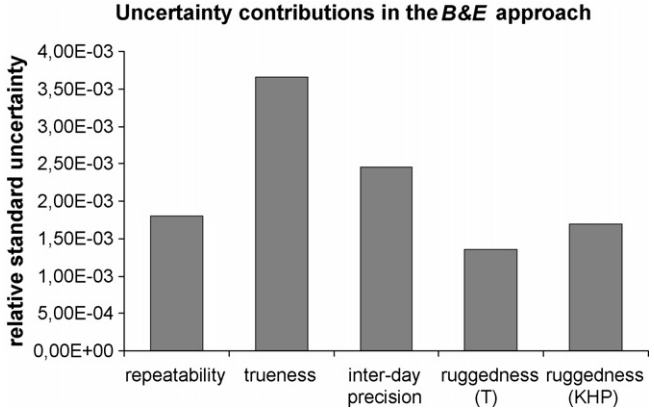

Fig. 3. Relative uncertainties in the *B&E* approach.

4. Conclusions

In this work three different approaches were employed to estimate the uncertainty in the chloroquine phosphate assay, a potentiometric titration reported in *European Pharmacopoeia* frequently used in medicinal control laboratories.

The three approaches delivered similar results. An expanded uncertainty of about 1% was assessed.

The first approach proposed was the ISO GUM *error-budget*. In the past it has been proven suitable for physical measurements but impractical for complex analytical chemistry methods such as LC, GC, etc. Many authors stressed this aspect noticing that the approach is too often complicated by a cumbersome algebra and is commonly prone to uncertainty underestimation [15–21].

The analytical method investigated in this work is far from being a complex method such the ones mentioned above, but nonetheless the calculations required to apply the GUM approach were rather heavy. Besides some effort was required to construct the Ishikawa diagram and assemble the error-budget itself; moreover a considerable care was needed and much time was spent in the proper evaluation of each source of uncertainty. Fortunately the Eurachem Guide [5] provided good examples of volumetric titrations that greatly helped in simplifying the whole process.

Thanks to this approach however a good insight into the *chloroquine* assay was gained: the estimation of each single source of uncertainty permitted to understand which step of the analytical procedure has to be handled with special care. For instance the approach showed that a temperature variation occurring between the determination of the $HClO_4$ titre and the *chloroquine* titre may result in an increased volumes uncertainty. This evidence suggested that a strict temperature control may result in a method performance improvement.

Another evident advantage of this approach, especially when compared to the others considered in this work, is the very low cost of its implementation. The only expenses sustained by the author's laboratory were those of the assay itself and of the quality assurance system, while no additional costs were required for the uncertainty studies. The ISO GUM approach in fact did not require any specifically designed experiment apart from those performed during the assay.

The second approach used to estimate the *chloroquine* assay uncertainty was the *top-down* of the *Analytical Methods Committee*. Its application resulted quite straightforward. A set of over 50 assay results obtained by the various European laboratories participating in the proficiency testing study mentioned in the text was at authors' disposal. Thus, the application of the *top-down* approach required just to follow a simple statistical process consisting of a few steps very clearly described in [8–10,36]. The main drawback of the approach resided in the expenses that the network of the European medicinal control laboratories sustained to set up the collaborative study. Moreover, the time needed to collect the various results was considerable. Another relevant aspect to bear in mind when applying this approach, as evidenced both in [11,41], is that it cannot be used to properly estimate uncertainty when bias and within-laboratory precision are not comparable in the various laboratories taking part into the collaborative study. In this work both aspects were under control since each laboratory had to fulfil a thorough method performance check according to the procedure reported in [32] before testing the sample under scrutiny.

The last approach employed in this work to estimate the uncertainty of the chloroquine titration was the B&E. This approach is based on the information gathered by a specifically designed method validation. The validation resulted not particularly troublesome since the method investigated was simple, a reference standard material was at authors' disposal and the various steps to be followed for the correct application of the approach were very well described in [39] where several examples have been of guidance. Nonetheless the effort spent by laboratory operators to accomplish the numerous analysis required by the B&E validation was ponderous, especially considering that no data were available from previous validations. In fact no validation at all is required by the *European Pharmacopoeia* for the chloroquine assay. Also the experimental design of the robustness tests required much time and care.

In conclusion ISO GUM, top-down and B&E approaches displayed a distinctive set of benefits and disadvantages. Although some difficulties were encountered relating most of all to procedural and experimental design, costs, organization and laboratory work, the practical implementation of all the three approaches to the investigated assay resulted feasible.

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