Evaluating measurement uncertainty in clinical chemistry

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Abstract

This report describes the ‘top-down’ approach to the evaluation of measurement uncertainty in the context of clinical chemistry. The ‘top-down’ approach makes use of information on the performance of whole measurement procedures, such as data obtained from routine internal quality control and external quality assessment. The strengths and weaknesses of the data commonly available to laboratories for uncertainty evaluation are discussed. The approach is illustrated by considering measurement procedures for the determination of creatinine in serum and catecholamines in urine.

Contents

1 Introduction 1

2 Accreditation requirements 2

2.1 ISO/IEC 17025 2

2.2 ISO 15189 3

2.3 CPA Standards for the medical laboratory 3

3 The ‘top-down’ approach to uncertainty estimation 3

3.1 Specify the measurand 4

3.2 Identify sources of uncertainty 4

3.3 Obtain an estimate of measurement precision 6

3.4 Obtain an estimate of measurement bias 7

3.5 Evaluate additional sources of uncertainty 11

3.6 Combine uncertainty estimates 12

3.7 Calculate the expanded uncertainty 12

4 Example 1: The determination of creatinine in serum 13

4.1 Background to the measurement procedure 13

4.2 Evaluation of measurement uncertainty 13

5 Example 2: The determination of free catecholamines in urine 19

5.1 Background to the method 19

5.2 Evaluation of measurement uncertainty 19

6 Conclusions 25

7 Acknowledgements 26

8 References 26

Appendix 1: Symbols and abbreviations 29

Appendix 2: Procedure for the determination of creatinine in serum 31

Appendix 3: Procedure for the determination of free catecholamines in urine 33

Appendix 4: Calculation of the uncertainty in the concentration of a calibration solution 37

1 Introduction

Laboratories carrying out all types of measurement are increasingly being asked to evaluate the uncertainties associated with their measurement results. For many laboratories, evaluating measurement uncertainty is now an accreditation requirement as specified by standards such as ISO/IEC 17025 [1], ISO 15189 [2] and, in the UK, CPA Standards for the Medical Laboratory [3].

Measurement uncertainty is defined in the ISO 'Guide to the Expression of Uncertainty in Measurement' [4] (often referred to as the 'GUM') as, 'a parameter, associated with the result of a measurement, that characterises the dispersion of the values that could reasonably be attributed to the measurand'.[‡]

An estimate of uncertainty provides a quantitative indication of the quality of a measurement result. Measurement procedures generally consist of many steps and require a range of equipment and reagents. The concentrations of reagents and calibrators will have uncertainties associated with them, and these uncertainties introduce uncertainties into the final measurement result obtained for a given sample. The operations carried out during the execution of a measurement procedure (e.g. measuring masses and volumes, instrumental measurements of absorbance or the integration of peak areas in a chromatogram) will also contribute to the uncertainty. Finally, the properties of the sample itself may also introduce uncertainties due to, for example, matrix effects or the presence of interferences. This means that, for any measurement, there will be not one but a range of values that could be reasonably attributed to the measurand. An estimate of uncertainty describes the dispersion of these values, combining the effect of all of the factors that influence the measurement result.

The fundamental principles for estimating measurement uncertainty are described in the GUM. The GUM has been interpreted for chemical measurements by Eurachem, in collaboration with CITAC [5]. There has been much discussion within the analytical community about how best to evaluate measurement uncertainty for results obtained in a routine testing environment. The GUM approach (often termed the 'bottom-up' approach to uncertainty evaluation) requires a mathematical model describing the measurement result which includes terms for all of the factors which can influence the result. These factors will be sources of uncertainty which will contribute to the uncertainty in the final result. An estimate of the magnitude of each source of uncertainty is obtained. These estimates are then combined to give the uncertainty in the measurement result. This approach is generally difficult to implement for chemical/biochemical measurements. The equations used to calculate measurement results will include parameters such as sample volumes or masses, instrument responses (absorbance readings, peak areas, etc) and the values of calibration standards but will not include other factors which are known to influence results (e.g. environmental conditions, concentrations of reagents, etc). The equation can be extended to include terms which represent these additional factors (indeed this can be helpful as part of the process of identifying sources of uncertainty). However, for many methods it is not practical to try to evaluate the individual uncertainty contributions for the factors that appear in the equation. Indeed, a recent study concluded that standard uncertainties obtained for chemical measurements using the GUM approach are likely to underestimate the measurement uncertainty [6].

Due to the issues associated with implementing the 'bottom-up' approach in a routine testing environment, approaches for evaluating measurement uncertainty for chemical/biochemical methods have focussed on using method performance data from method validation studies, internal quality control (IQC) and external quality assessment (EQA) [7-13]. Evaluating measurement uncertainty using such data is often referred to as

[‡] The measurand is the 'quantity intended to be measured' (see section 3.1).

the ‘top-down’ approach. Whereas the ‘bottom-up’ approach examines the *inputs* to a method and considers how they might influence results, the ‘top-down’ approach uses information from method *outputs* (e.g. the observed variability of replicate measurement results). The ‘top-down’ approach has a number of advantages for testing laboratories. For example, existing data are used thus minimising the amount of additional experimental work, and calculations are simplified as one set of data (e.g. data from the replicate analysis of a quality control material) will account for a number of sources of uncertainty.

This report outlines the ‘top-down’ approach to uncertainty estimation and discusses how it can be applied in clinical analysis. Measurement procedures in clinical chemistry fall into two broad categories – measurements made using automated chemical analysers and those made using analytical techniques such as HPLC. The ‘top-down’ approach is illustrated by the evaluation of the measurement uncertainty for two clinical chemistry measurement procedures – the determination of creatinine in serum (using a chemical analyser) and free catecholamines in urine (a high performance liquid chromatography method). The current accreditation requirements are also discussed.

2 Accreditation requirements

The accreditation standards most relevant to clinical laboratories are ISO 15189 and, in the UK, the CPA Standards for the Medical Laboratory. The requirements of ISO/IEC 17025 are also discussed in this section as this standard is widely used in testing laboratories in other sectors and is a normative reference in ISO 15189.

2.1 ISO/IEC 17025

This standard specifies requirements for both calibration and testing laboratories. The requirements described in this report relate to testing laboratories. The requirements for calibration laboratories are more stringent as the values and uncertainties reported for the measurement standards they produce will contribute to the uncertainties in measurement results obtained by testing laboratories using the standards. The evaluation of measurement uncertainty by testing laboratories is covered by clauses 5.4.6.2 and 5.4.6.3 of ISO/IEC 17025. Clause 5.4.6.2 states that ‘Testing laboratories shall have and shall apply procedures for estimating uncertainty of measurement’ and that ‘…the laboratory shall at least attempt to identify all the components of uncertainty and make a reasonable estimation, and shall ensure that the form of reporting the result does not give a wrong impression of the uncertainty.’ The clause also states that existing data, such as method validation data can be used when evaluating uncertainty: ‘Reasonable estimation shall be based on knowledge of the performance of the method and on the measurement scope and shall make use of, for example, previous experience and validation data.’

Clause 5.4.6.3 covers the uncertainty components which should be included in an uncertainty estimate: ‘all uncertainty components which are of importance…shall be taken into account…’ Note 1 to this clause lists a number of sources which contribute to the uncertainty: reference standards and reference materials, methods and equipment, environmental conditions, properties and condition of the item being tested, the operator.

Clause 5.10.3.1 covers the content of test reports. Laboratories are required to include a statement of the estimated uncertainty ‘where applicable’. Reporting the uncertainty is necessary when it is relevant to the validity or application of the test results, when a customer’s instruction requires it or when the uncertainty affects compliance to a specification limit.

The United Kingdom Accreditation Service (UKAS) has published general guidance on how laboratories can meet the uncertainty of measurement requirements in ISO/IEC 17025 [14].

2.2 ISO 15189

Measurement uncertainty is addressed in ISO 15189 section 5.6 on assuring the quality of examination procedures. Laboratories are required to 'determine the uncertainty of results, where relevant and possible' (clause 5.6.2). Important sources of uncertainty must be taken into account and a number of possible sources of uncertainty are listed (sampling, sample preparation, sample portion selection, calibrators, reference materials, input quantities, equipment used, environmental conditions, condition of the sample, changes of operator).

Section 5.8 covers the reporting of results. Clause 5.8.3 states that information on uncertainty of measurement 'should be provided on request'.

2.3 CPA Standards for the medical laboratory

The CPA standards are aligned with the requirements of ISO 15189. Measurement uncertainty is covered in section F3 (assuring the quality of examinations). Paragraph F3.3 states that 'The laboratory shall determine the uncertainty of results where relevant and possible.'

The CPA has issued a guidance document for assessors describing how requirements for measurement uncertainty should be interpreted during laboratory assessments [15]. The guidance treats measurement uncertainty as the imprecision (i.e. the spread) of results due to random effects. The measurement uncertainty is estimated as $2s$ where s is a standard deviation which estimates the imprecision. The document does not consider systematic effects, which result in biased results, to be part of the measurement uncertainty. However, laboratories are required to evaluate the bias. It should be noted that this approach is not in line with the ISO definition which considers uncertainty arising from both systematic and random effects as contributing to measurement uncertainty.

The CPA standards and guidance document do not require the measurement uncertainty to be reported with measurement results. However, there should be 'evidence that actions are taken to eliminate, reduce to a minimum or take into account any uncertainty of measurement when interpreting results.' [15]

3 The 'top-down' approach to uncertainty estimation

The requirements in ISO/IEC 17025 (see section 2.1) make it clear that an uncertainty estimate can use existing method performance data, for example from method validation studies. There are a number of papers and guides which describe the use of method performance data in the evaluation of measurement uncertainty for clinical measurement procedures [16-24].

The 'top-down' approach is discussed in a number of documents including the Eurachem/CITAC Guide [5], the Eurolab report on alternative approaches to uncertainty evaluation [12] and ISO 21748 [13]. The latter focuses on the use of data from interlaboratory studies for method validation. The key steps in evaluating measurement uncertainty using the top-down approach are outlined below and discussed in more detail in the following sections:

1. Write down a clear description of the measurand;
2. Study the measurement procedure in detail and identify all the possible sources of uncertainty;
3. Obtain an estimate of the precision of the measurement procedure;
4. Obtain an estimate of the measurement bias and the uncertainty associated with the bias estimate;

5. Evaluate any sources of uncertainty identified in step 2 which are not adequately covered by the precision and bias data;
6. Express the uncertainty estimates obtained in steps 3-5 in an appropriate form and combine using rules for the combination of variances;
7. Multiply the combined uncertainty obtained in step 6 by a suitable 'coverage factor' to obtain an uncertainty estimate with the required level of confidence.

3.1 Specify the measurand

The measurand is defined in the International Vocabulary of Metrology (VIM) as the 'quantity intended to be measured' [25]. 'Quantity' is defined as the 'property of a phenomenon, body, or substance, where the property has a magnitude that can be expressed as a number and a reference'. Examples of quantities include mass, volume, length, and amount-of-substance concentration of a given entity.

The term 'analyte' is frequently used in analytical science to describe the element/compound being determined using a particular measurement procedure. However, 'analyte' should not be used as an alternative to 'measurand' as a description of the analyte does not refer to a quantity. Identification of the analyte is only one part of the specification of the measurand for an analytical measurement. As a minimum, specification of the measurand should include the particular quantity to be measured (e.g. amount-of-substance concentration), the analyte (e.g. lead) and the tissue in which the analyte is to be measured (e.g. blood). In some cases it will be necessary to include further information in the specification of the measurand. For example if the measurand can only be defined with reference to an agreed standard measurement procedure then information on that measurement procedure (e.g. a reference to the standard method) should be included. It is also important to state whether the measurand relates to an individual sample 'as received in the laboratory', or to the patient. If it is the latter then 'pre-analytical' steps (see Table 1) associated with sampling, sample storage and transport, etc. will contribute to the uncertainty.

3.2 Identify sources of uncertainty

It is important to be clear about what the uncertainty in the measurement result is intended to represent as this will influence the sources of uncertainty that need to be considered. The steps in the process of producing a measurement result for a particular patient sample - from obtaining the sample to interpretation of the result – can be grouped into a number of distinct stages. These general stages are often referred to as 'pre-analytical', 'analytical' and 'post-analytical'[‡] as illustrated in Figure 2 and Figure 4 which show, respectively, the key steps in the procedures for the determination of creatinine in serum and catecholamines in urine (see sections 4 and 5 for further information on the procedures). In general, it is relatively easy for laboratories to identify and assess the sources of uncertainty associated with the analytical steps as these are the aspects of the process over which the laboratory has direct control. Some of the pre-analytical steps may occur within the laboratory (for example preparation of samples prior to loading on an analyser), but collection and storage of the samples prior to submission for analysis is outside of the laboratory's direct control.

The processes described in this report will focus on the uncertainties associated with the analytical steps. The NPAAC [16] and AACB [19] documents consider measurement uncertainty as encompassing only the factors which occur during the measurement procedure (i.e. the analytical stages). However, the NPAAC document states that laboratories should have standard operating procedures in place to reduce pre-analytical sources of uncertainty (such as sample collection techniques, sample storage and

[‡] Note: the term 'examination' is frequently used in place of 'analytical', for example in ISO 15189.

transportation) to acceptable levels and the AACB guide recognises that it is important to 'identify and minimise' pre- and post-analytical factors.

The CPA guidance document [15] identifies a number of sources of uncertainty associated with pre- and post-examination stages. The document states that laboratories should take steps to reduce uncertainties associated with pre-examination stages. The guidance recognises that it may not be possible for laboratories to quantify the uncertainties associated with all stages of a measurement process, but a laboratory should be able to provide evidence that they have accounted for them. Linko et al [18] and Rynning et al [26] have discussed the evaluation of sources of uncertainty that arise from non-analytical steps.

Examples of sources of uncertainty associated with different stages in a measurement process are given in Table 1 [collated from references 2, 15, 16, 27]. It should be noted that an uncertainty estimate is intended to reflect the performance of a measurement procedure when it is under statistical control. Possible gross errors due to, for example, instrument failure or clear deviations from method protocols are not therefore considered as sources of uncertainty.

Table 1: Examples of sources of uncertainty

Pre-analytical
Patient state (e.g. fasting vs. non-fasting)
Biological variation
Patient preparation
Time of sample collection
Site of sample collection
Sampling technique
Method of collection (e.g. tube additives)
Sample transport
Sample storage (e.g. time, temperature)
Analytical
Preparation of (sub)sample for analysis
Sample properties (e.g. sample matrix, interfering compounds)
Environmental conditions (temperature, humidity)
Calibrators and calibration of instruments (including calibrator values, batch-to-batch differences between calibrators and commutability of reference materials)
Factors relating to the measuring instrument, including measurement precision
Factors relating to the measurement (e.g. recovery of analyte from sample matrix, calibration function, blank correction, reaction times, absorbance readings)
Reagent composition (including batch-to-batch variation)
Volume of sample and reagents
Analyst
Post-analytical
Software, including algorithms
Reporting of results
Interpretation of results

One approach to identifying sources of uncertainty for a particular measurement procedure is to use 'cause and effect' analysis. This involves capturing the possible sources of uncertainty associated with each stage of the measurement process on a cause and effect or 'fishbone' diagram. The process of constructing and using cause and effect diagrams in uncertainty evaluation is discussed in detail in the Eurachem/CITAC guide [5]. Cause and effect diagrams for the creatinine and catecholamines procedures are shown in Figure 3 and Figure 5, respectively. Note that these diagrams cover only the analytical stages of the procedures and that there has been no 'refinement' of the diagrams to remove duplicate terms or effects that would cancel.

3.3 Obtain an estimate of measurement precision

The top-down approach to uncertainty estimation requires a sound estimate of the precision of the measurement process. Measurement precision is defined as, 'closeness of agreement between indications or measured quantity values obtained by replicate measurements on the same or similar objects under specified conditions' [25]. Precision is usually expressed numerically by measures of *imprecision* such as a standard deviation or relative standard deviation (coefficient of variation). Random variation in the individual steps of a measurement process causes the observed dispersion of measurement results. When evaluating measurement uncertainty, the aim is therefore to obtain a precision estimate which captures as many of the sources of random variation associated with the measurement process as possible. A parameter which can be shown to have varied representatively during the period over which precision data were obtained requires no further study when evaluating uncertainty. Two sources of information are commonly used for the evaluation of method precision:

- data from the analysis of quality control materials (internal quality control (IQC) data);
- data from method validation studies.

These options are discussed in more detail in sections 3.3.1 and 3.3.2.

The precision should be studied at appropriate analyte concentrations. It may therefore be necessary to obtain data at more than one analyte level. If data are obtained at different levels a decision will be required on how best to represent the precision of the method. Ideally, a single uncertainty estimate will be obtained which can be applied to all test samples which fall within the method scope. There are three scenarios which may be encountered.

a) Precision is independent of analyte concentration: The standard deviations calculated from data obtained at different analyte concentrations are not significantly different. In this case the precision can be expressed as a standard deviation.

b) Precision proportional to analyte concentration: The relative standard deviations calculated from data obtained at different analyte concentrations are not significantly different. In this case the precision can be expressed as a relative standard deviation.

c) No simple relationship between precision and analyte concentration: For some methods, particularly if a wide range of analyte concentrations are being measured, there may be no straightforward relationship between the precision and the analyte concentration. In such cases separate precision estimates (and therefore separate uncertainty estimates) will be required to cover different analyte concentrations.

The issue of level dependence is discussed in the Eurachem/CITAC guide [5].

3.3.1 Using IQC data to evaluate precision

For laboratories operating well-established measurement procedures, data from the analysis of quality control materials can provide a valuable source of data for the evaluation of method precision. The data should be collected over a number of months so that factors such as reagent/calibrator batch, operator and instrument condition (maintenance state) will have varied. For well-established methods in regular use the AACB document recommends using a minimum of 6 months' QC data [19].

An estimate of measurement uncertainty is intended to indicate the range of values which can be attributed to the measurand when the measurement process is operating correctly. A measurement uncertainty estimate will not include the effect of gross errors (e.g. instrument failure, deviations from standard operating procedures, transcription/calculation errors, etc). Care should therefore be exercised when deciding which IQC data to use in an uncertainty calculation. Over time, there will be QC failures (usually detected by the application of 'Westgard rules' [28]). Such 'QC failures' generally lead to the rejection of test results and the reanalysis of samples. The precision estimate for uncertainty evaluation should therefore be based on QC data obtained when the measurement procedure was considered to be under statistical control.

If replicate QC measurements are made within a run, it is possible to calculate the within- and between-run variation in results using analysis of variance (ANOVA) [29]. This is illustrated in the creatinine worked example (see section 4).

The use of IQC data makes the assumption that the behaviour of the QC materials in the measurement process is similar to that of test samples. If there are any stages of the measurement procedure that are not applied to QC materials then the uncertainties associated with those stages will need to be evaluated separately (see section 3.5).

3.3.2 Using method validation data to evaluate precision

If the measurement uncertainty is required for results from a new method for which no or limited QC data are available an alternative estimate of the precision is required. This can be obtained from studies carried out during method validation/verification. The aim during validation is to obtain an indication of the likely precision once a measurement procedure is put into routine use. A validation study should therefore consider run-to-run variation in measurement results in addition to within-run precision. Khatami et al describe a protocol for assessing precision during measurement verification [30]. The protocol recommends analysing one series of samples per day for a minimum of five days. The series should consist of five replicate samples at two or more concentrations. The materials to be analysed during the precision study could be reference materials (e.g. IQC materials) or prepared from pooled patient samples to give samples with clinically relevant concentrations of the analyte. To simulate normal operating conditions, factors such as operator and reagent/calibrator batch should ideally be varied between series. As mentioned above, when evaluating precision as part of a measurement uncertainty study, as many parameters as possible should be varied during the course of the precision experiments. ANOVA can be used to evaluate the within- and between-series variation [29].

3.4 Obtain an estimate of measurement bias and its uncertainty

Precision provides an estimate of the variability in results from one measurement to the next. A precision study does not provide any information on how close results are to the true value of the measurand. Since an uncertainty estimate is intended to represent a range of values within which the true value lies, a complete study of measurement uncertainty must include an evaluation of measurement bias.

Bias is defined as an 'estimate of a systematic measurement error' [25]. It is estimated experimentally by calculating the difference between the mean of a set of measurement results and a suitable reference value, either as an absolute value or as a percentage:

$$\text{Bias} = \bar{x} - x_0 \qquad \text{Eq. 1a}$$

$$\%\text{Bias} = \frac{\bar{x} - x_0}{x_0} \times 100 \qquad \text{Eq. 1b}$$

where $\bar{x}$ is the mean of the set of measurement results and x_0 is the reference value.

The most common approaches used for evaluating measurement bias are:

- analysis of a certified reference material (CRM) – x_0 is the value assigned to the CRM;
- analysis of a reference material prepared in-house (e.g. by spiking a suitable sample matrix) – x_0 is the value assigned to the reference material;
- results obtained from participation in an EQA scheme – x_0 is the value assigned to the EQA material by the scheme organiser.

These options are discussed in more detail in sections 3.4.1 to 3.4.3. Note that the reference value used to assess measurement bias must be independent of any reference value used for calibration, i.e. it is not acceptable to use the same batch of a CRM to calibrate a measurement procedure and to assess the measurement bias.

As with a precision study, evaluation of measurement bias should cover a representative range of sample types as specified by the scope of the measurement procedure. The resulting bias estimates will require evaluation to determine whether a single value can be applied to all samples or whether separate estimates (and therefore separate uncertainty estimates) are required. It is also important to identify which stages of the measurement process are covered by the bias estimate. Any steps that are not covered will need separate evaluation (see section 3.5).

3.4.1 Estimation of bias using certified reference materials

Certified reference materials[‡] provide a traceable reference value. If a suitable material exists (i.e. a material that is similar to test samples in terms of sample composition and analyte concentration) it should be the first choice when assessing measurement bias. However, the number of certified reference materials available which meet the internationally agreed definition of a CRM is relatively small compared with the wide range of sample/analyte combinations encountered in the laboratory. The COMAR database [31] contains information on reference materials for all areas of measurement. The Joint Committee for Traceability in Laboratory Medicine (JCTLM) maintains a database of higher-order reference materials [32]. The values of reference materials in the JCTLM database are traceable either to SI units (e.g. values for electrolytes, drugs, metabolites and non-peptide hormones) or to an internationally agreed protocol (e.g. reference materials for blood typing, coagulation factors and nucleic acids).

3.4.2 Estimation of bias from the analysis of spiked samples

In this case the laboratory prepares an in-house reference material by adding a known amount of the analyte of interest to a suitable, previously analysed, sample matrix. The concentration of the analyte in the spiked material is calculated and this gives the reference value for the bias study. Spiked materials do not always respond to

[‡] A certified reference material (CRM) is defined as a 'reference material, accompanied by documentation issued by an authoritative body and providing one or more specified property values with associated uncertainties and traceabilities, using valid procedures' [25].

measurement procedures in the same way as genuine test materials. Care must be taken to ensure that the added analyte has time to equilibrate with the sample matrix before the analysis is carried out.

3.4.3 Evaluation of bias using data from EQA

For well established methods it may be possible to obtain an estimate of measurement bias from results obtained from the participation in EQA. A key feature of EQA is the 'target' or 'assigned' value for the EQA material. This is the value that participants' results are compared with and it can therefore be considered a type of reference value. However, care needs to be taken when assessing bias by comparison with EQA assigned values. The target value is set by the scheme organiser and in many cases is based on a consensus obtained from participants' results (after processing the data to reduce the influence of extreme values). Consensus values should be interpreted with caution. When bias is calculated using consensus values from EQA schemes the outcome is a bias relative to the results obtained by other participants rather than a bias against a traceable 'true' value. For well established measurement processes the consensus obtained from participants' results will often be a good estimate of the true value but this will not be the case for all methods. An added complication is that EQA data are often processed on a method-by-method basis with different target values for different methodologies. This would provide a bias estimate relative to results from laboratories using the same type of method.

However, EQA data can provide a useful indication of measurement bias, as long as users are aware of the limitations of this type of data. For many laboratories it may well be the only information that is readily available. A number of papers and guides discuss the use of EQA data in relation to assessing bias; see for example references 16, 18, 19, and 26.

3.4.4 Evaluating the uncertainty associated with a bias estimate

As shown in Eq. 1a, bias is simply the difference between the mean of laboratory observations ($\bar{x}$) and a reference value (x_0). Since there will be an uncertainty associated with both $\bar{x}$ and x_0, there will also be an uncertainty associated with the estimated bias. If $u(\bar{x})$ and $u(x_0)$ are the standard uncertainties[‡] in $\bar{x}$ and x_0, respectively, the standard uncertainty in the bias $u(B)$ is (see section 3.6 for information on combining uncertainties):

$$u(B) = \sqrt{u(\bar{x})^2 + u(x_0)^2} \qquad \text{Eq. 2}$$

The evaluation of $u(\bar{x})$ and $u(x_0)$ will depend on how the bias has been evaluated. If a CRM has been analysed, $u(\bar{x})$ is generally the standard deviation of the mean of the measurement results and $u(x_0)$ is obtained from the certificate accompanying the CRM.

In the case of spiked materials $u(\bar{x})$ is also generally the standard deviation of the mean of results obtained from the analysis of spiked materials and $u(x_0)$ can be calculated from information on uncertainties associated with the stages in preparing the material.

Obtaining an estimate of the uncertainty associated with the bias estimate can be more complicated if it is based on the results obtained from the participation in EQA rounds. For each EQA material, the reference value used to calculate the bias will be the assigned value (X) for the material. In a single EQA round a participating laboratory would obtain only limited information as each EQA material is typically analysed only once. However, over several rounds a number of estimates of the bias will be obtained. These can be used to calculate a mean bias and an estimate of the uncertainty in the mean bias (this is typically the standard deviation of the mean of the bias estimates). It should also be possible to obtain an estimate of the uncertainty in the assigned value for each EQA

[‡] A standard uncertainty is a 'measurement uncertainty expressed as a standard deviation' [25].

material. The standard ISO 13528 [32] describes approaches to calculating the uncertainty in an assigned value. The approach used will depend on how the assigned value was obtained. In EQA schemes for clinical chemistry one of the most common approaches for determining assigned values is to use a consensus value obtained from the participants' results. Typically the consensus is a robust average of the results reported by all participants in the round. A robust average, such as the median of the results, is used to reduce the influence of extreme values. If a robust average and standard deviation of participants' results have been calculated according to the algorithm given in Annex C of the standard, the standard uncertainty in the assigned value, $u(X)$, is estimated as:

$$u(X) = 1.25 \times \frac{s^*}{\sqrt{p}} \qquad \text{Eq. 3}$$

where s^* is the robust standard deviation and p is the number of participant results used to calculate the assigned value and the robust standard deviation.[‡]

When using results from several EQA rounds to evaluate method bias there will be different assigned values with different associated uncertainties for each round. In this case the EUROLAB Technical Report [12] recommends calculating the average of the estimates of $u(X)$ to obtain a typical value. This is the approach followed in this report but it should be noted that this gives an approximate value for the uncertainty, as taking an average of standard deviations gives a biased estimate. Taking the average of the uncertainties expressed as variances and then taking the square root would provide a more statistically rigorous estimate.

The uncertainty in the mean bias estimate is obtained by combining the uncertainty in the mean bias with the estimate of the uncertainty in the assigned value, using the 'square root of the sum of squares' rule, as in Eq. 2.

3.4.5 Include bias and its uncertainty in the uncertainty estimate

Once the bias and its uncertainty have been evaluated, the final step is to consider how any bias should be accommodated in the uncertainty estimate. The approach taken will depend on whether the bias is considered to be significant. For an unbiased method the average bias, over a large number of measurements, would be equal to zero. To determine whether the observed measurement bias is significantly different from zero, the uncertainty associated with the bias estimate must be taken into account. To give the required level of confidence, the standard uncertainty in the bias estimate is usually multiplied by a factor of 2 to give an expanded uncertainty $U(B)$ (see section 3.7) at a confidence level of approximately 95%. If the range $B \pm U(B)$ includes zero, the bias is considered not statistically significant. There are three general scenarios which may be encountered:

1. The bias is not significantly different from zero;
2. The bias is significant and results are corrected to take account of the known bias;
3. The bias is significant but for operational reasons results are not corrected.

Each of these is discussed below. The issue of incorporating bias into uncertainty estimates has been studied in detail by Magnusson and Ellison [34], and O'Donnell and Hibbert [35].

[‡] ISO 13528 contains a note explaining that the factor 1.25 represents the ratio of the standard deviation of the median to the standard deviation of the arithmetic mean, for large samples (p>10) from a normal distribution. For robust averages which are not a straightforward median, the standard deviation of the average will be somewhere between the standard deviation of the arithmetic mean and the standard deviation of the median. The formula will therefore give a conservative estimate of the standard uncertainty in the assigned value.

Case 1: The bias is not significantly different from zero. In this case it is recommended that bias is assumed to be equal to zero with an uncertainty equal to $u(B)$. The uncertainty $u(B)$ is included in the uncertainty estimate.

Case 2: The bias is significant and results are corrected to take account of the known bias. In this case the corrected measurement result is reported and the uncertainty associated with the correction $u(B)$ is included in the uncertainty estimate. This is the approach described in the GUM, which assumes that any known biases have been corrected for and that the uncertainties associated with making the corrections have been included in the uncertainty estimate.

This approach is uncommon in chemical analysis – historically results have not been corrected to take account of measurement bias (case 3).

Case 3: The bias is significant but for operational reasons results are not corrected. Ideally measurement methods should be developed to remove significant biases but this is not always technically possible. If no allowance is made for a known significant bias when reporting results and the associated uncertainty, a simple report of the measurement uncertainty is likely to mislead. One acceptable option is to evaluate the uncertainty for uncorrected results (i.e. excluding any uncertainty associated with the bias) and to document the bias and its uncertainty separately. Using this approach users of the results are not being misled and can correct results themselves if necessary.

Where a separate report of the bias is not feasible, some authorities recommend increasing the reported uncertainty so that it includes the corrected result [36]. The Eurolab guide also describes an approach whereby the bias and its uncertainty is included in the uncertainty estimate by calculating the 'root mean square' of the bias [12]. References 34 and 35 discuss the options in detail.

3.5 Evaluate additional sources of uncertainty

Any factors which may influence the measurement result but have not been adequately covered by the precision or bias data require separate consideration. These additional sources of uncertainty will vary from method to method and will also depend on the data used to evaluate precision and bias. Factors to consider may include:

- sample pre-treatment steps which were not applied to the materials used to evaluate precision or bias;
- sample stability;
- matrix effects (e.g. if composition of QC materials does not adequately represent the range of sample types analysed);
- calibrator uncertainty (e.g. if the same calibrator lot was used during the precision and bias studies).

Information on how to evaluate additional sources of uncertainty can be found in the Eurachem/CITAC guide [5].

3.6 Combine uncertainty estimates

Uncertainties are combined using the square root of the sum of squares rule. The basic rule is illustrated in Figure 1.

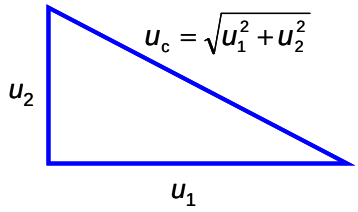

Figure 1: Basic rule for combining uncertainties. u_1 and u_2 are independent uncertainty contributions expressed as standard deviations and u_c is the combined uncertainty.

In the figure, u_1 and u_2 are uncertainties expressed as standard deviations (standard uncertainties). In some cases it is appropriate to combine uncertainty contributions as relative values rather than as standard deviations. If relative values are combined the resulting combined uncertainty will also be a relative value. The GUM [4] and Eurachem/CITAC guide describe the rules for combining uncertainties in detail [5].

3.7 Calculate the expanded uncertainty

Measurement uncertainty is generally reported as an expanded uncertainty. An expanded uncertainty is obtained by multiplying a standard uncertainty by an appropriate coverage factor *k*. The coverage factor used depends on the level of confidence required. In most cases a coverage factor $k = 2$ is used which gives an expanded uncertainty at approximately the 95% confidence level.

4 Example 1: The determination of creatinine in serum

4.1 Background to the measurement procedure

The procedure discussed in this example is an *in vitro* diagnostic test used to measure creatinine in human serum. The creatinine result is used, along with the patient's age, sex and ethnic origin, to calculate the estimated glomerular filtration rate (eGFR) which is an indicator of chronic kidney disease (CKD).

The analysis is carried out using a chemical analyser. The system uses a creatinine reagent system which utilises a bilirubin oxidant that rapidly and completely oxidises bilirubin prior to reaction monitoring thus making it free from bilirubin interference. The reaction is measured kinetically to virtually eliminate interferences from Jaffe positive substances.

Creatinine reacts with picrate ions in an alkaline solution to form a red tautomer of creatinine picrate which can be quantified spectrophotometrically at 500 – 530 nm.

$$\text{Creatinine + picric acid} \xrightarrow{^{-}\text{OH}} \text{Creatinine-picrate complex (red)}$$

An outline of the measurement procedure is given in Appendix 2. Figure 2 shows the key stages of the procedure.

4.2 Evaluation of measurement uncertainty

The uncertainty associated with results for creatinine in serum was evaluated following the procedure outlined in section 3.

4.2.1 State the measurand

The measurand is the amount-of-substance concentration of creatinine in human serum (expressed as μmol L^{-1}).

4.2.2 Identify sources of uncertainty

The cause and effect diagram in Figure 3 identifies the possible uncertainties associated with the procedure for the determination of creatinine. Note that only the uncertainties associated with the analytical stages of the method have been considered (see Table 1). Dashed lines have been used for the branches relating to sample dilution as this stage in the method is only required for samples containing greater than 1400 μmol L^{-1} creatinine.

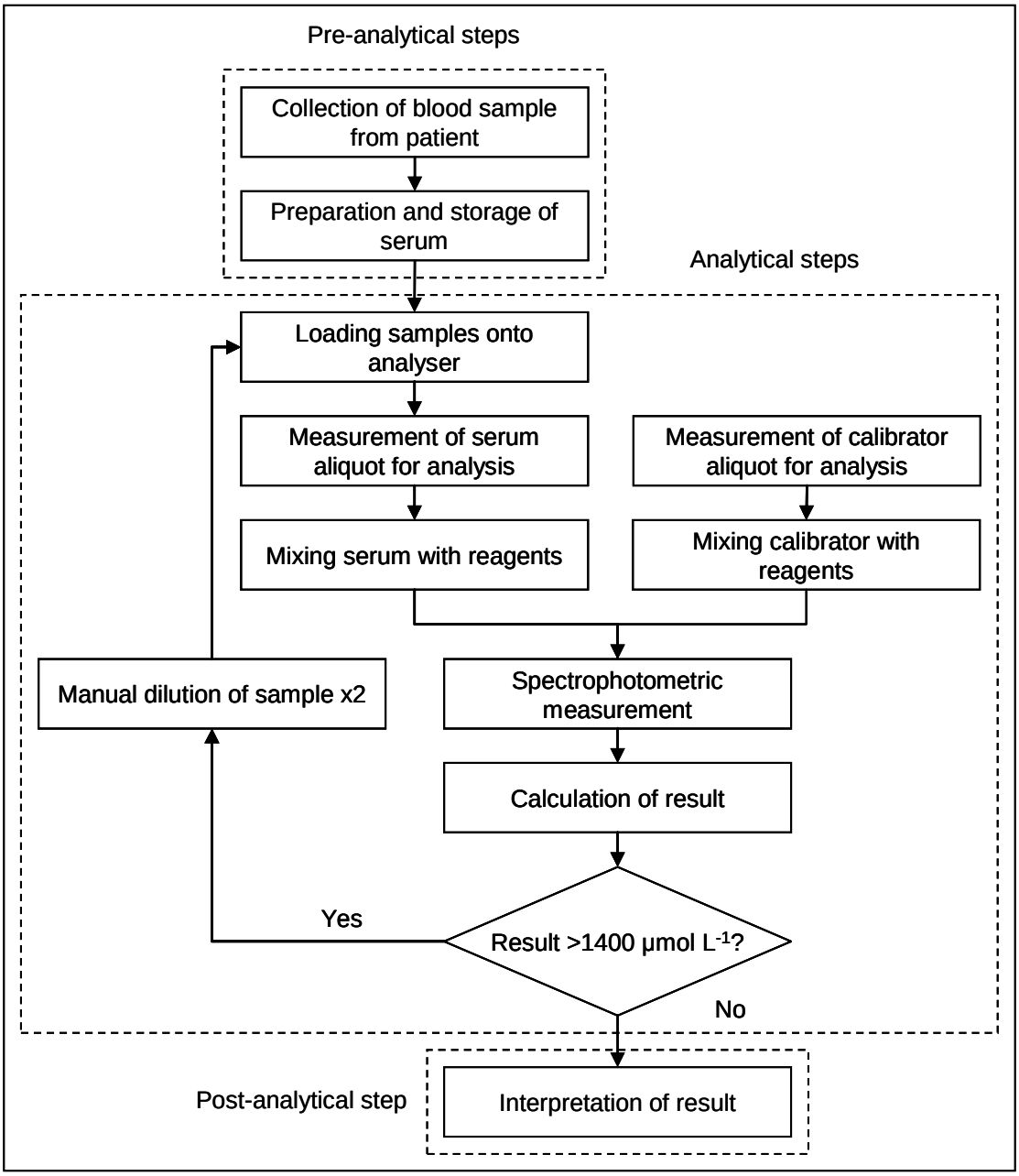

Figure 2: Key steps in the measurement procedure for the determination of the concentration of creatinine in serum samples.

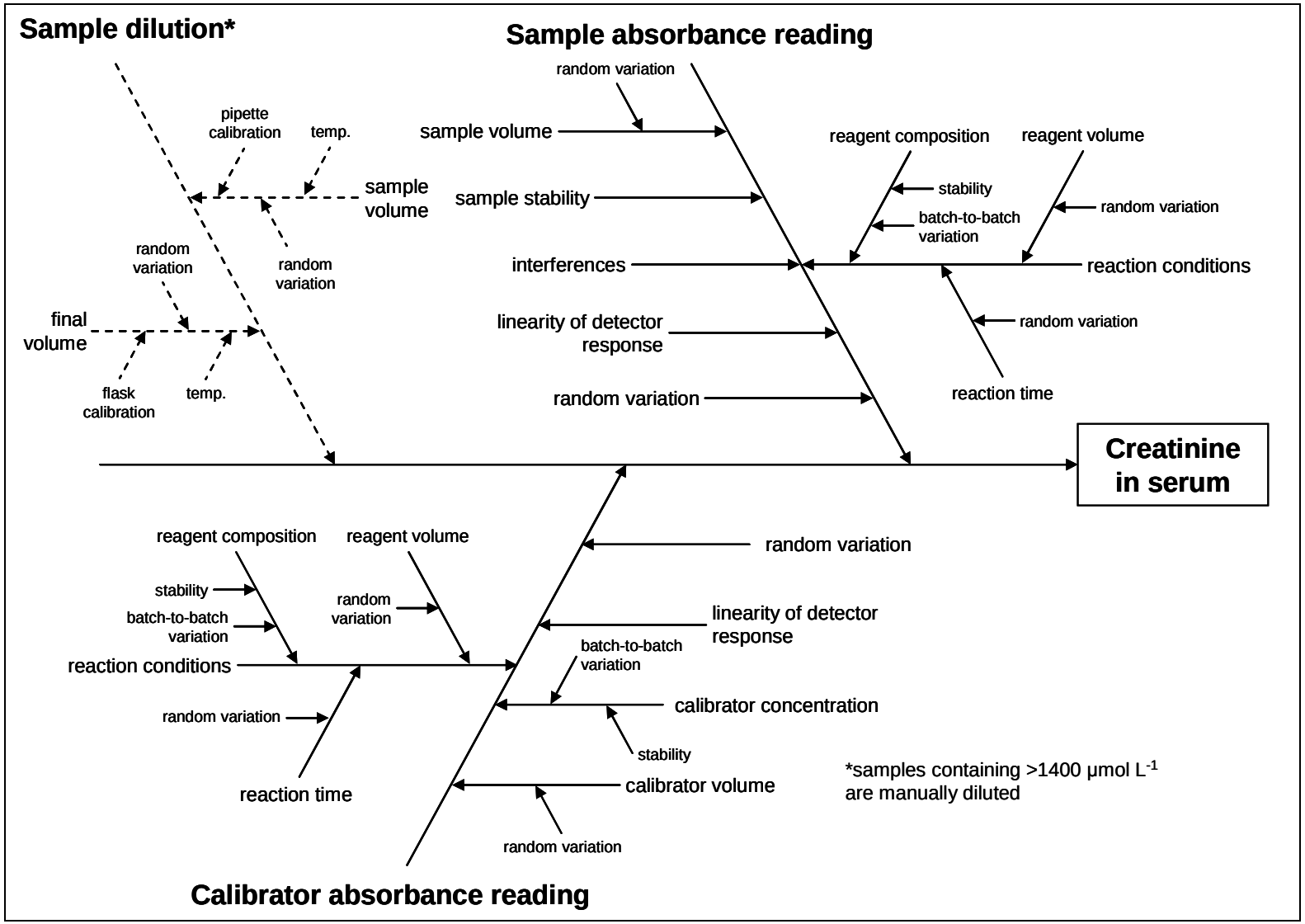

Figure 3: Cause and effect diagram identifying possible sources of uncertainty associated with the determination of creatinine in serum.

4.2.3 Obtain an estimate of the precision of the measurement procedure

Three quality control materials are analysed regularly alongside patient samples. The QC materials are commercially supplied liquid controls prepared from human serum. The values shown in Table 2 were calculated from data obtained over a 5 month period. QC results that were rejected as 'out of specification'[‡] have been omitted from the calculations.

Table 2: Summary of results from the analysis of QC materials for the determination of creatinine in human serum

	Quality control material		
	1	**2**	**3**
number of results	543	548	543
mean of results (µmol L^{-1})	75.9	176.6	524.3
within-day standard deviation (µmol L^{-1}) [(a)(b)]	1.4	2.3	6.4
within-day %CV	1.9	1.3	1.2
between-day component of variation (expressed as a standard deviation) (µmol L^{-1})[(b)]	1.0	1.7	5.3
between-day component of variation (%CV)	1.3	1.0	1.0
total standard deviation (µmol L^{-1})[(c)]	1.7	2.8	8.3
total %CV	2.3	1.6	1.6

[(a)]All standard deviations and %CV have been rounded to 2 significant figures.

[(b)]Within- and between-day terms estimated via analysis of variance [29].

[(c)]Total standard deviation is calculated as the square root of the sum of the squares of the within- and between-day terms.

The data show that there is a significant between-day component of variation in the results. The precision estimate should therefore be based on the total standard deviation which includes both the within-day and between-day components of variation. The total standard deviation clearly varies with creatinine level so it is not appropriate to use a single estimate of the standard deviation for all levels. When expressed as a coefficient of variation (%CV) the total precision is consistent for materials QC2 and QC3, but the precision is poorer at the lower creatinine concentration. If the laboratory requires a single precision estimate then the laboratory could use the largest value (2.3%) as the 'worst case' estimate for all samples within the concentration range studied. An alternative is to use separate precision estimates for different creatinine levels, for example 2.3% for samples with concentrations below 150 µmol L^{-1} and 1.6% for samples with concentrations greater than 150 µmol L^{-1}. In this study the second option was chosen.

4.2.4 Obtain an estimate of the measurement bias and its uncertainty

The only information available for this method was from the regular participation in EQA. Table 3 summarises the results obtained over a 6 month period (two distributions per month, three samples per distribution). The %bias is calculated for the result for each sample as follows:

[‡] Using the mean and standard deviation previously established for the QC materials, results >3 standard deviations from the mean are rejected.

$$\%\text{bias} = \frac{x - X}{X} \times 100$$

where x is the laboratory's result and X is the target (assigned) value for the sample set by the scheme organiser. The target value is a consensus value obtained from results from participants using the same method.

Table 3: Summary of results from participation in EQA for the determination of creatinine in serum over a 6 month period

number of results	39
max concentration of EQA materials (μmol L^{-1})	587.4
min concentration of EQA materials (μmol L^{-1})	50.9
mean %bias	-3.0
standard deviation of the mean of %bias	0.51
95% confidence interval for %bias	-3.0±1.0

The report provided to the laboratory by the EQA scheme organiser for each distribution gives the bias for each sample and a 'B score'. The B score in UK NEQAS schemes is the average %bias calculated in a rolling time window. Before the mean is calculated, the individual %bias values are ranked and the highest and lowest outliers trimmed to provide a more robust estimate of the mean. The EQA scheme organiser specifies an acceptable B score of ±10% for the determination of creatinine. At the end of the 6 month time period covered in this study the laboratory's B score was -3.4%.

The bias estimates were obtained over a range of creatinine concentrations (51 to 587 μmol L^{-1}). There was no clear relationship between bias and creatinine concentration. The mean %bias and the individual %bias values were all within the ±10% specified by EQA scheme organiser. However, since the precision is to be reported for two different concentration ranges (see section 4.2.3), the bias and its uncertainty was also calculated for the same ranges.

For samples with concentrations below 150 μmol L^{-1} the mean bias was -2.6% with a standard deviation of the mean of 0.63. The uncertainty in the assigned value for each sample was calculated using Eq. 3. Following the approach outlined in the Eurolab document [12] the average uncertainty in the assigned value, expressed as a %CV, was calculated as 1.0. Combining the uncertainty in the mean bias with the uncertainty in the assigned value gives an uncertainty in the %bias of:

$$u(\%B) = \sqrt{0.63^2 + 1.0^2} = 1.2$$

The expanded uncertainty in the bias estimate is 2.4, (calculated using a coverage factor k = 2 to give a confidence level of approximately 95%).

For samples with concentrations greater than 150 μmol L^{-1} the mean bias was -4.3% with a standard deviation of the mean of 0.42. The uncertainty in the assigned value was estimated as 0.81. The uncertainty in the mean %bias is therefore 0.91 (the expanded uncertainty is 1.8, k=2).

The mean bias for both concentration ranges would be considered statistically significant as the expanded uncertainty for the bias estimate does not include zero. In chemical measurement, the current recommended approach for dealing with significant bias is to document the bias and its uncertainty separately from the rest of the uncertainty budget. If the measurement uncertainty is included when results are reported to end users then the bias and its uncertainty must also be reported.

The main weakness with the bias estimates obtained from the EQA data is that the target values used are consensus values which are calculated on a method-by-method basis.

The bias estimates of -2.6% and -4.3% therefore gives an indication of how the laboratories results compare with those produced by other laboratories using the same measurement procedure. However, the reference ranges established for the clinical interpretation of results are defined for particular measurement systems, so bias calculated on a method specific basis is relevant in this case.

4.2.5 Evaluate any sources of uncertainty not adequately covered by the precision and bias data

The precision and bias data were obtained over several months. The data would therefore be expected to reflect the majority of sources of variability associated with the measurement procedure such as:

- random variation in the volume of sample and calibrator delivered by the analyser;
- variation in time elapsed between mixing the sample and calibrator with the reagents and measuring the absorbance;
- stability of the reagents;
- changes in reagent lot;
- stability of samples and the calibrator;
- interferences from other components of the sample;
- random variation in the absorbance measurement of the sample and calibrator;
- change in operator.

No information was available on the uncertainty in the concentration the calibrator. For this uncertainty to make a significant contribution to the combined uncertainty, it would need to have a value greater than one-third of the largest uncertainty component.

4.2.6 Combine the uncertainty estimates and calculate the expanded measurement uncertainty

Since the bias is statistically significantly different from zero, it is documented separately, along with its uncertainty. Since the precision and bias data are considered to have accounted for all significant sources of variability the uncertainty is as follows:

Creatinine concentration (μmol L^{-1})	Relative standard uncertainty (%)	Relative expanded uncertainty (%)*	Bias (%)	%Bias standard uncertainty
<150	2.3	4.6	-2.6	1.2
>150	1.6	3.2	-4.3	0.91
*Expanded uncertainty calculated using a coverage factor *k* = 2 to give a confidence level of approximately 95%.				

5 Example 2: The determination of free catecholamines in urine

5.1 Background to the method

This example describes a procedure for the determination of adrenaline, noradrenaline and dopamine in urine which uses high performance liquid chromatography (HPLC) with electrochemical detection.

1 mL of urine, QC material or calibration solution, plus the internal standard, is diluted with phosphate buffer. After pH adjustment the solution is passed through a solid phase extraction (SPE) system. The resulting acidic eluate is analysed by HPLC.

An outline of the measurement procedure is given in Appendix 3. Figure 4 shows the key stages of the procedure.

5.2 Evaluation of measurement uncertainty

The uncertainty associated with results for adrenaline, noradrenaline and dopamine in urine was evaluated following the procedure outlined in section 3.

5.2.1 State the measurand

The measurands are the amount-of-substance-concentration of adrenaline, noradrenaline and dopamine in urine (expressed as nmol L^{-1}).

5.2.2 Identify sources of uncertainty

The cause and effect diagram in Figure 5 identifies the possible uncertainties associated with the procedure for the determination of catecholamines. Note that only the uncertainties associated with the analytical stages of the method have been considered (see Table 1).

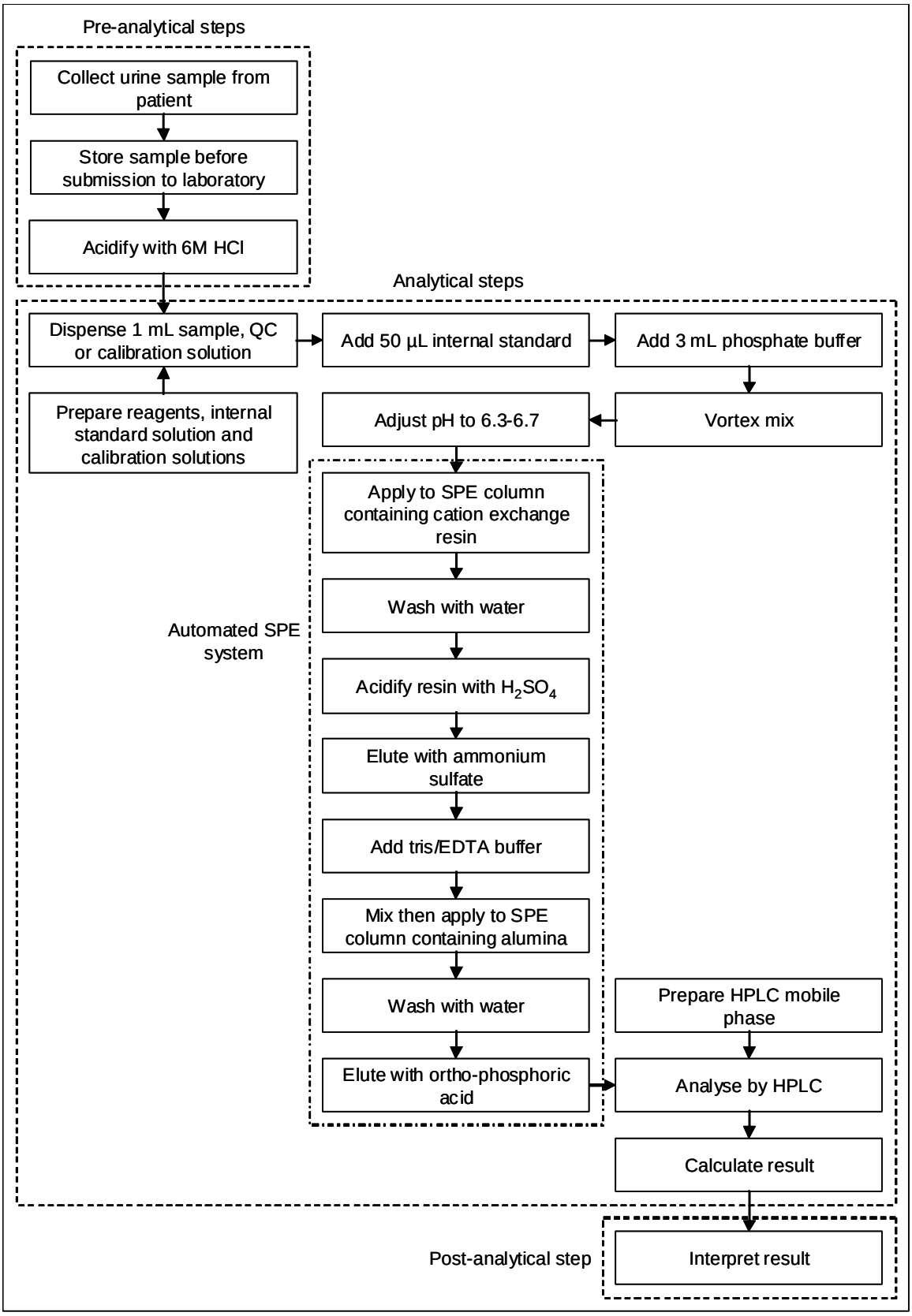

Figure 4: Key steps in the measurement procedure for the determination of free catecholamines in urine samples.

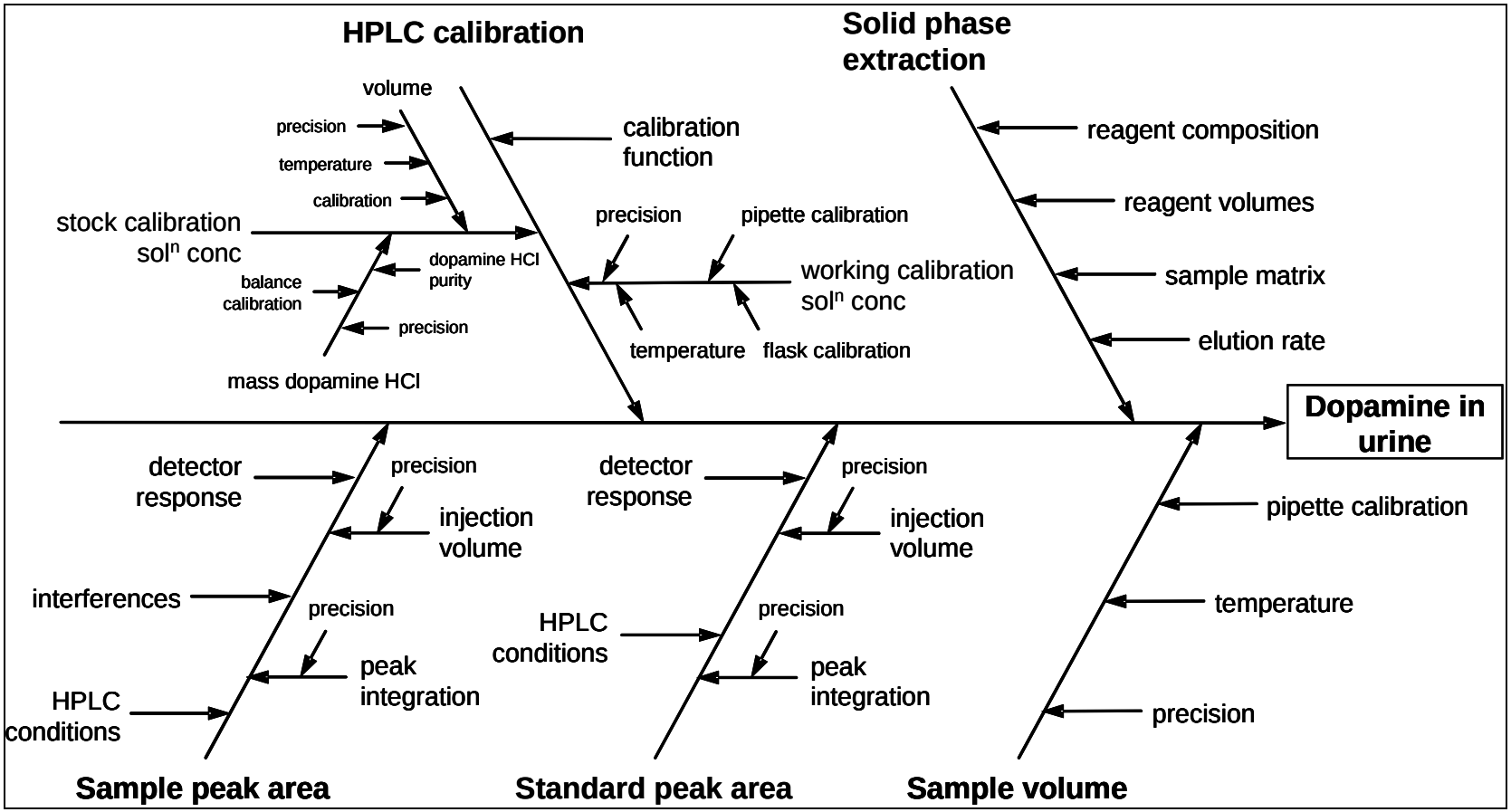

Figure 5: Cause and effect diagram identifying possible sources of uncertainty associated with the determination of catecholamines in urine (the example shown is for dopamine; similar diagrams would apply for adrenaline and noradrenaline).

5.2.3 Obtain an estimate of the precision of the measurement procedure

Data from the analysis of quality control materials

Two materials are analysed with each assay. Typically, two assays are performed per week. A summary of results obtained over a 9 month period is presented in Table 4.

Table 4: Summary of results from the analysis of QC materials (results expressed in nmol L^{-1})

		noradrenaline			adrenaline			dopamine		
QC material	*n*	mean	*s*	%CV	mean	*s*	%CV	mean	*s*	%CV
QC1 (Low)	75	207	22.8	11	68	9.5	14	481	43.3	9
QC2 (Medium)	75	854	85.4	10	404	52.5	13	2413	265.4	11

For each analyte there was no significant difference between the %CV obtained for the analysis of QC1 and QC2. It is therefore acceptable to use the average %CV for each analyte:

Analyte	**Average %CV**
noradrenaline	11
adrenaline	13
dopamine	10

5.2.4 Obtain an estimate of the measurement bias and its uncertainty

The only information available for this method was from the regular participation in EQA. Table 5 summarises the results obtained from the participation in an EQA scheme. The results were obtained over a 10 month period (one distribution per month, three samples per distribution). The % bias was calculated from:

$$\%\text{bias} = \frac{x - X}{X} \times 100$$

where x is the laboratory's result and X is the target (assigned) value set by the scheme organiser. The target value is a consensus value obtained from results from participants using methods based on HPLC with electrochemical detection.

Table 5: Summary of results from participation in EQA over a 10 month period

	noradrenaline	adrenaline	dopamine
number of results	24	22	24
max conc. of EQA materials (nmol 24 hr^{-1}*)	798	390	2517
min conc. of EQA materials (nmol 24 hr^{-1})	122	104	643
mean %bias	-9.2	-9.7	-12.9
standard deviation of the mean of %bias	1.6	2.8	2.4
95% confidence interval for %bias	-9.2±3.3	-9.7±5.9	-12.9±5.0
*Urine volume is assumed to be 1.0 L collected over a 24 hr period.			

As mentioned in section 4.2.4, the EQA scheme organiser supplies a 'B score' for each analyte. The acceptable B score for noradrenaline, adrenaline and dopamine is ±25%. The laboratory B scores at the end of the 10 month period are shown below:

Noradrenaline: -6.3%

Adrenaline: -9.7%

Dopamine: -7.9%

The bias estimates were obtained over a range of analyte concentrations. There was no clear relationship between bias and analyte concentration.

The uncertainty in the assigned value for each sample was calculated using Eq. 3. Following the approach outlined in the Eurolab document [12] the average uncertainty in the assigned value for each analyte, expressed as a %CV, was calculated:

Noradrenaline: 1.1

Adrenaline: 1.4

Dopamine: 1.1

Combining the standard deviation of the mean for each bias estimate (see Table 5) with the mean uncertainty for the assigned value, using the square root of the sum of the squares rule give the standard uncertainties shown below.

	Mean %bias	%Bias standard uncertainty	%Bias expanded uncertainty
Noradrenaline	-9.2	2.0	3.9
Adrenaline	-9.7	3.2	6.3
Dopamine	-12.9	2.7	5.4

In all cases the bias is clearly significant as the expanded uncertainty for the bias estimate does not include zero. In chemical measurement, the current recommended approach for dealing with significant bias is to document the bias and its uncertainty separately from the rest of the uncertainty budget. Ideally, a laboratory should seek to adjust its measurement procedures to remove the effect of significant bias but this is not always technically feasible. In this case the bias, although significant, is within the performance target set by the EQA scheme organiser,

The main weakness with the bias estimates obtained from the EQA data is that the target values used are consensus values which are calculated on a method-by-method basis. The bias therefore gives an indication of how results compare with those produced by other laboratories using HPLC with electrochemical detection. However, reference ranges established for the clinical interpretation of results are defined for particular measurement systems so laboratory bias, calculated on a method specific basis, is relevant in this case.

5.2.5 Evaluate any sources of uncertainty not adequately covered by the precision and bias data

The precision and bias data were obtained over several months. The data would therefore be expected to reflect the majority of sources of variability associated with the measurement procedure such as:

- preparation of reagents;
- stability of reagents;
- volumes of reagents;
- preparation of SPE columns;

- preparation of internal standard solution;
- preparation of diluted and working calibration solutions;
- HPLC parameters;
- change in operator.

The stock calibration solution may not have been replaced during the period in which the precision and bias data were obtained as the measurement procedure (see Appendix 3) specifies that the solution is stable for up to 1 year when stored at 4 ^{o}C. The uncertainty associated with the concentration of the stock calibration solution is unlikely to be significant compared to the precision and bias but as an illustration, an example of the calculation of the uncertainty is given below.

The concentration of the solution in mmol L^{-1} is calculated from:

$$C = \frac{m \times P}{v \times M} \times 1000 \qquad \text{Eq. 4}$$

where m is the mass of the compound (mg), P is its purity (expressed as a ratio between 0 and 1), v is the final volume of the solution (mL) and M is the molecular mass of the compound (g mol^{-1}).

The calculation is illustrated for the uncertainty in the concentration of dopamine in the stock calibration solution. A standard uncertainty is required for each of the parameters in Eq. 4. The data are given in Table 6. Further information on how the individual uncertainty components were evaluated is given in Appendix 4.

Table 6: Uncertainty data for the calculation of the uncertainty in the concentration of dopamine in the stock calibration solution

Description	**Value**	**Standard uncertainty**	**Comment**
Mass of dopamine hydrochloride	18.96 mg	0.0234 mg	Standard uncertainty calculated from information on balance calibration certificate and standard deviation of replicate weighings of a check weight.
Purity of dopamine hydrochloride	1 (Expressed as a ratio. No correction for purity applied)	0.0050	Standard uncertainty calculated from supplier's purity information.
Volume of solution	100 mL	0.0621 mL	Standard uncertainty calculated from manufacturer's specification for flask, 'fill-and-weigh' data for filling flask, temperature effects.
Molecular mass of dopamine hydrochloride	189.64 g mol^{-1}	0.00392 g mol^{-1}	Standard uncertainty calculated from IUPAC data on atomic weights.

The concentration of dopamine in the stock calibration solution is:

$$\frac{18.96 \times 1.0}{100 \times 189.64} \times 1000 = 1.0 \text{ mmol L}^{-1}$$

The standard uncertainties for each parameter are combined as relative values:

$$u(C) = C \times \sqrt{\left(\frac{u(m)}{m}\right)^2 + \left(\frac{u(P)}{P}\right)^2 + \left(\frac{u(v)}{v}\right)^2 + \left(\frac{u(M)}{M}\right)^2}$$

$$u(C) = 1.0 \times \sqrt{\left(\frac{0.0234}{18.96}\right)^2 + \left(\frac{0.0050}{1.0}\right)^2 + \left(\frac{0.0621}{100}\right)^2 + \left(\frac{0.00392}{189.64}\right)^2}$$

$$u(C) = 1.0 \times \sqrt{1.52 \times 10^{-6} + 2.50 \times 10^{-5} + 3.86 \times 10^{-7} + 4.27 \times 10^{-10}} = 0.00519 \text{ mmol L}^{-1}$$

The concentration of dopamine in the stock calibration solution is therefore 1.0±0.005 mmol L^{-1} (a relative uncertainty of 0.5%). This is clearly insignificant compared with the precision and bias estimates (10% precision and -13±2.7 %bias for dopamine).

5.2.6 Combine the uncertainty estimates and calculate the expanded measurement uncertainty

Since the bias is statistically significantly different from zero, it is documented separately, along with its uncertainty. However, if the measurement uncertainty is included when results are reported to end users then the bias and its uncertainty must also be reported. Since the precision and bias data are considered to have accounted for all significant sources of variability the uncertainty is as follows:

Analyte	Relative standard uncertainty (%)	Relative Expanded uncertainty (%)*	Bias (%)	%Bias standard uncertainty
Noradrenaline	11	22	-9.2	2.0
Adrenaline	13	26	-9.7	3.2
Dopamine	10	20	-12.9	2.7
*Expanded uncertainty calculated using a coverage factor *k* = 2 to give a confidence level of approximately 95%.				

6 Conclusions

The 'bottom-up' approach to uncertainty estimation has been found to be difficult to implement in routine testing laboratories. An alternative 'top-down' approach, which makes use of method validation and other method performance data, has therefore been developed. This approach has been widely applied in many sectors. This report has focused on the evaluation of measurement uncertainty in the clinical sector, specifically clinical chemistry. In this sector there is generally a significant amount of IQC and EQA data available but there may be limited in-house method validation data. This is especially true for measurands determined using automated chemical analysers. These measurement procedures fall under the scope of the *in vitro* diagnostic (IVD) directive [37]. The analyser, reagents, calibrators and measurement procedure are supplied by the manufacturer and used, without modification, by the laboratory. The manufacturer is required to validate the system to demonstrate that it is fit for the intended use. However, the laboratory is required to verify that it can meet the manufacturer's performance claims before the measurement procedure is introduced. This process is generally less detailed than a full method validation. In this report we have therefore focused on the use of IQC and EQA data in uncertainty evaluation. Data gathered from the analysis of QC materials over a number of months should provide a good estimate of the long term random variability of measurement results. Parameters that have varied

representatively during the period that the data were gathered do not require further evaluation of their contribution to the uncertainty estimate.

While it should be relatively easy for laboratories to obtain an estimate of measurement precision, obtaining an estimate of measurement bias can be more problematic. Ideally, bias should be assed using a certified reference material which is representative of patient samples. Unfortunately the availability of suitable reference materials is limited compared to the wide range of analytes and sample matrices. For many laboratories, the only data available which gives an indication of bias comes from participation in EQA schemes. In the majority of EQA schemes, the 'assigned value' used to calculate bias is a consensus obtained from participants' results. In addition, the assigned value is often calculated on a method-by-method basis so the bias in an individual laboratory's result will, in effect, be relative to results produced by laboratories using the same measurement procedure. For well established measurement processes the consensus obtained from participants' results is often not significantly different from the true value but this will not be the case for all methods.

Another issue facing laboratories when evaluating uncertainty is that the values of the calibrators supplied by instrument manufactures often do not have uncertainties associated with them. Generally, such uncertainties would be expected to be small compared to measurement bias and precision. However this should ideally be confirmed by contacting the manufacturer and requesting information on the uncertainty associated with calibrator values.

This report has illustrated how laboratories can use IQC and EQA data to provide an estimate of measurement uncertainty that should be fit for purpose in a routine testing environment, as long as the limitations of the data are understood.

7 Acknowledgements

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Appendix 1: Symbols and abbreviations

Symbols

α	coefficient of volume expansion
B	measurement bias
C	amount-of-substance concentration
CV	coefficient of variation
k	coverage factor used in the calculation of expanded uncertainty
m	mass of material
n	number of values in a data set
M	molecular mass
p	number of participant results in an EQA round
P	purity of a pure substance reference material
s	sample standard deviation
*s**	robust estimate of standard deviation
u	standard measurement uncertainty
$u(x_i)$	uncertainty associated with a value x_i
U	expanded measurement uncertainty
$\bar{x}$	sample mean
x_0	reference value (e.g. value associated with a certified reference material)
x	laboratory result submitted to EQA scheme
X	assigned value in EQA scheme

Abbreviations

AACB	Australian Association of Clinical Biochemists
ANOVA	analysis of variance
CITAC	Co-operation on International Traceability in Analytical Chemistry
CKD	chronic kidney disease
COMAR	international database for certified reference materials
CPA	Clinical Pathology Accreditation (UK) Ltd
CRM	certified reference material
eGFR	estimated glomerular filtration rate
EDTA	ethylenediaminetetraacetic acid
EQA	external quality assessment
Eurachem	European network of analytical laboratories
Eurolab	European federation of national associations of measurement, testing and analytical laboratories
GUM	Guide to the Expression of Uncertainty in measurement (ISO Guide 98-3)

HPLC	high performance liquid chromatography
IEC	International Electrotechnical Commission
IQC	internal quality control
ISO	International Organization for Standardization
JCTLM	Joint Committee for Traceability in Laboratory Medicine
NPAAC	National Pathology Accreditation Advisory Council (Australia)
SI	Système International (d'Unitès) (International System of Units)
SPE	solid phase extraction
UKAS	United Kingdom Accreditation Service
UK NEQAS	United Kingdom National External Quality Assessment Service
VIM	International Vocabulary of Metrology (ISO Guide 99)

Appendix 2: Procedure for the determination of creatinine in serum

Note that the method description has been simplified for this case study. It is not intended to represent a standard operating procedure for use in the laboratory.

1. Method scope

1.1 Sample type: Serum

Creatinine is stable in the sample for 7 days at room temperature.

1.2 Assay range: 0–1900 μmol L^{-1}

1.3 Samples giving results >1400 μmol L^{-1} should be manually diluted x2 and reanalysed.

2. Reagents

2.1 Reagent 1

Reagent 1a: Creatinine buffer, contains sodium hydroxide (0.17 mol L^{-1}) with non-reactive surfactants and solvents.

Reagent 1b: Oxidant, contains potassium ferricyanide and preservatives.

Storage: 20–25 °C, protected from light.

Stability: Until the expiration date on the pack label, when unopened.

Preparation: Add the entire contents of one vial of oxidant (Reagent 1b) to one bottle of Buffer (Reagent 1a).

Storage: 20–25 °C, protected from light.

Stability: 7 days after preparation.

2.2 Reagent 2: Creatinine picrate, contains picric acid (25 mol L^{-1})

Preparation: Reagent is ready to use and requires no preparation.

Storage: 20–25 °C, protected from light.

Stability: Until the expiration date on the pack label, when unopened.

3. Assay calibration

3.1 SETpoint™ calibrator

The supplier states a 'Principal Assigned Value' for the calibrator of 730 μmol L^{-1} (established by HPLC using the American Association for Clinical Chemistry reference method).

Preparation: Reconstitute with 3 mL of reagent water. Manually mix and invert 10 times every 10 minutes for a period of 30 minutes, or until reconstitution is complete. Prior to use, mix by inversion at least 5 times to ensure homogeneity.

Storage: 2–8 °C.

Stability: Until the expiration date on the vial when unopened, 48 hours once opened.

3.2 Calibration procedure

The creatinine assay requires calibration:

- daily, following morning maintenance and setup;
- when changing reagent container;

- when changing reagent lot number;
- when quality control results are repeatedly out of range.

4. Quality control

4.1 Quality control material

Multiqual 1, 2 and 3 Quality Control Material, for serum.

Preparation: Allow to stand at room temperature for 1 hour or until completely thawed. Before sampling, gently swirl the contents until homogeneous with no visible signs of precipitate.

Storage: -20 to -70 °C. Once thawed store at 2-8 °C.

Stability: Until expiration date when stored unopened at -20 to -70 °C. Stable for 30 days when stored unopened at 2-8 °C or for 14 days when stored opened and tightly capped at 2-8 °C.

4.2 Quality control procedure

To monitor the system and chart trends, the quality control materials should be run:

- daily, following morning setup and maintenance;
- every 4 hours;
- each time the assay is calibrated;
- on advice of service engineers, following periodic maintenance and service.

5. Assay procedure

5.1 Samples can be loaded onto the chemistry analyser either manually or via the automated track system.

5.2 Dilutions: All samples giving results >1400 µmol L^{-1} should be manually diluted, x2 and reanalysed to obtain an accurate result.

6. Calculation and interpretation of results

Results are calculated by the instrument software on the basis of a comparison of the absorbance change for the sample and calibrator over a set period of time.

Appendix 3: Procedure for the determination of free catecholamines in urine

Note that the method description has been simplified for this case study. It is not intended to represent a standard operating procedure for use in the laboratory.

1. Method scope

1.1 Sample type: Urine

Samples should be stored at 4 °C and acidified with 6M HCl on receipt in the laboratory.

Samples are stable for 1 month at 4 °C.

2. Reagents

2.1 0.1M phosphate buffer, pH 7.0

2.2 0.7M sulfuric acid

2.3 2M ammonium sulphate

2.4 3M tris/EDTA buffer, pH 8.0

2.5 0.1M orthophosphoric acid

2.6 0.5M sodium hydroxide

2.7 Resin generation buffer (1M phosphate buffer, pH 6.25)

2.8 Resin diluent buffer (0.1M phosphate buffer, pH 6.25)

3. Preparation of SPE column packing

3.1 Weakly acidic cation exchange resin

Wash approximately 250 g resin several times with dionised water to remove fines.

Suspend in 4-5 volumes of 0.5M sodium hydroxide (2.6) and mix for approximately 10 minutes with a paddle stirrer. Allow resin to settle and pour off supernatant liquid.

Wash 3-4 times with dionised water.

Suspend resin in approximately 2 volumes of 1M phosphate buffer (2.7) and mix with a paddle stirrer. Adjust pH to 6.25 using 10M sodium hydroxide or concentrated orthosphosphoric acid. Discard the supernatant and replace with fresh buffer (2.7). Repeat pH adjustment until pH remains constant at 6.25 on changing the buffer. Discard the supernatant ad re-suspend the resin in 1-2 volumes of 0.1M phosphate buffer (2.8).

Store at 4 °C.

3.2 Alumina

Wash approximately 50 g alumina as follows:

3 times with 5 volumes of 0.1M orthophosphoric acid (2.5)

5 times with 5 volumes of deionised water.

Pour off the water and dry alumina completely in an oven. Store in a screw-top bottle.

4. Preparation of HPLC mobile phase

4.1 Stock HPLC phosphate buffer	Dissolve the following in approximately 1600 mL deionised water:
	276 g $NaH_2PO_4.1H_2O$ (equivalent to 1 mol L^{-1})
	24.3 g 1-heptane sulfonic acid (equivalent to 60 mmol L^{-1})
	2.98 g Na_2EDTA (equivalent to 4 mmol L^{-1})
	Adjust the pH to 3.0 using concentrated orthophosphoric acid.
	Make up to 2 L in a volumetric flask.
	Stability: 3 weeks at room temperature.
4.2 HPLC mobile phase	Dilute 100 mL of stock phosphate buffer (4.1) to 2 L with deionised water using a measuring cylinder. Add 75 mL acetonitrile. Filter before use.

5. Internal standard and calibration solutions.

5.1 Internal standard (IS) solution

5.1.1 Stock IS solution (nominal concentration 1.0 mmol L^{-1})	Dissolve 22.01 mg 3,4 dihydroxybenzylamine hydrobromide (3,4 DHBA) in 0.1M HCl and make up to 100 mL in a volumetric flask
	Stability: 6 months when stored at 4 °C
5.1.2 Working IS solution (nominal concentration 5 µmol L^{-1})	Dilute 0.5 mL stock solution (5.1.1) to 100 mL with 0.1M HCl
	Stability: 6 months when stored at 4 °C

5.2 Calibration solutions

5.2.1 Stock calibration solutions (nominal concentration 1.0 mmol L^{-1})

Adrenaline	Dissolve 21.97 mg adrenaline HCl in 0.1M HCl and make up to 100 mL in a volumetric flask
	Stability: 1 year when stored at 4 °C
Noradrenaline	Dissolve 20.56 mg noradrenaline HCl in 0.1M HCl and make up to 100 mL in a volumetric flask
	Stability: 1 year when stored at 4 °C
Dopamine	Dissolve 18.96 mg dopamine HCl in 0.1M HCl and make up to 100 mL in a volumetric flask
	Stability: 1 year when stored at 4 °C

5.2.2 Prepare a diluted stock calibration solution for each analyte with a nominal concentration of 10.0 µmol L^{-1} by diluting the stock calibration solutions (5.2.1) with 0.1M HCl (1 mL of stock solution diluted to 100 mL).

Stability: 1 month or every 4 assays (whichever is sooner) when stored at 4 °C.

5.2.3 Prepare a single working top calibration solution by dispensing the specified volumes of the diluted stock calibration solutions (5.2.2) into a 100 mL volumetric flask and making up to volume with 0.1M HCl.

	Vol. diluted stock	Conc. in top calibration standard
Adrenaline	2 mL	200 nmol L^{-1}
Noradrenaline	4 mL	400 nmol L^{-1}
Dopamine	20 mL	2000 nmol L^{-1}

Stability: Store at 4 °C, make up fresh for each assay.

5.2.4 Prepare two further working calibration solutions with the following concentrations, by diluting the working top calibration solution (5.2.3) with 0.1M HCl:

	Adrenaline	Noradrenaline	Dopamine
Working calibration solution 1 (nmol L^{-1})	50	100	500
Working calibration solution 2 (nmol L^{-1})	100	200	1000

Stability: Store at 4 °C, make up fresh for each assay.

6. Quality control materials

Low and medium controls reconstituted with 10 mL 0.01M HCl. Values are lot dependent.

Analyse QC materials with each batch of samples.

7. Preparation of samples, QC materials and calibration standards for analysis

Carry out the following procedure for each sample, the two QC materials and the three working calibration solutions:

- using an automatic pipette dispense 1 mL of sample/QC material/calibration standard into a glass tube;
- using an automatic pipette add 50 µL working internal standard solution (5.1.2);
- using a pipette add 3 mL 0.1M phosphate buffer (2.1);
- mix on vortex mixture;
- adjust pH to 6.3-6.7 using either 25% H_3PO_4 or 2M NaOH.

Solid phase extraction (SPE) clean-up:

- apply solution to cation exchange resin column;
- wash with water;
- acidify resin with H_2SO_4;
- elute with ammonium sulphate;
- add tris/EDTA buffer to eluate;
- apply to alumina column;

- wash with water;
- elute with orthosphosphoric acid.

After clean-up the samples, QC materials and calibration solutions are analysed by HPLC with electrochemical detection.

8. Assay calibration

Working standards are freshly prepared and analysed for each assay.

Linear regression is carried out to establish the calibration function for each analyte (the response used in the linear regression is the peak are ratio obtained for the analyte peak and the internal standard peak).

9. Calculation of results

The concentration of adrenaline, noradrenaline and dompamine in each sample is calculated from the calibration function.

Appendix 4: Calculation of the uncertainty in the concentration of a calibration solution

The method for the determination of catecholamines in urine requires the preparation of a stock calibration solution (see Appendix 3, section 5.2.1). The calculations required to evaluate the uncertainty in the concentration of a solution are outlined below, using the preparation of the dopamine stock calibration solution as an example.

The solution is prepared by dissolving 18.96 mg dopamine hydrochloride in 0.1M HCl and making up to volume in a 100 mL volumetric flask. The concentration of the solution in mmol L^{-1} is calculated from:

$$C = \frac{m \times P}{v \times M} \times 1000$$

where m is the mass of dopamine hydrochloride (mg), P is its purity (expressed as a ratio between 0 and 1), v is the final volume of the solution (mL) and M is the molecular mass of the dopamine hydrochloride (g mol^{-1}).

To calculate the uncertainty in the concentration of the solution, a standard uncertainty is required for each of the parameters in the equation.

Mass of the dopamine hydrochloride

The calibration certificate for a typical 5-figure balance states an uncertainty ±0.00003 g (expanded uncertainty at the 95% confidence level, calculated using a coverage factor $k = 2$). Dividing the expanded uncertainty by the stated coverage factor gives a standard uncertainty of 0.015 mg. The precision of the balance was estimated as 0.018 mg (replicate weighings of a 10 mg check weight over a 1 month period). Combining these values using the square root of the sum of the squares rule gives a standard uncertainty in the mass of dopamine hydrochloride of 0.023 mg.

Purity of dopamine hydrochloride

The purity of the dompamine used to prepare the solution is quoted by the supplier as 99%. No information on the uncertainty associated with the purity value is given. When the concentration of the solution is calculated the purity of the dopamine is not taken into account (i.e. the value of P in Eq. 4 is assumed to be one). This assumption adds a small uncorrected bias to the concentration of the solution. As a first estimate, the uncertainty associated with the purity was taken as the difference between the actual purity (0.99) and the assumed purity (1.0) divided by the coverage factor, $k = 2$ (assuming a confidence level of 95%). Treating the uncertainty in this way ensures that the expanded uncertainty for the concentration of the solution includes the value that would be obtained if the purity was corrected for.

Volume of the solution

Three components contribute to the uncertainty in the volume of the liquid in the 100 mL volumetric flask:

i) the uncertainty in the stated internal volume of the flask;

ii) the precision in filling the flask to the calibration line;

iii) the flask and solution temperature differing from the flask calibration temperature.

The manufacturing tolerance for a Class A 100 mL volumetric flask is ±0.1 mL. For specifications of this type a rectangular distribution is normally assumed. The standard deviation for a rectangular distribution is obtained by dividing the stated tolerance (i.e. the half-width of the distribution) by √3. The standard uncertainty associated with the stated internal volume of the flask is therefore 0.1/√3 = 0.0577 mL.

The uncertainty associated with the precision of filling the flask to the calibration line is estimated by performing a series of 'fill-and-weigh' experiments. The standard deviation of the results from ten such experiments was 0.0173 mL.

The effect of the temperature differing from the flask calibration temperature can be calculated from an estimate of the variation of the laboratory temperature around the calibration temperature and the coefficient of volume expansion of the liquid. Since the coefficient of volume expansion of the glass is much smaller than that of the liquid, only the latter needs to be considered. The possible temperature variation around the calibration temperature of 20 ℃ is estimated as ±3 ℃ (with 95% confidence). This 95% confidence interval is converted to a standard uncertainty by dividing by 2 (the approximate Student *t* value for a large number of degrees of freedom). The coefficient of volume expansion for aqueous liquids is approximately $1x10^{-4}$ $℃^{-1}$. The uncertainty due to temperature variation $u(v_T)$ is calculated from:

$u(v_T) = v \times \alpha \times u(T)$

where v is the volume of the liquid, α is the coefficient of volume expansion and $u(T)$ is the standard uncertainty in the temperature.

In this example, the standard uncertainty in the volume due to temperature effects is therefore:

$u(v_T) = 100 \times 1x10^{-4} \times 1.5 = 0.015$ mL.

The three uncertainty components described above are combined using the square root of the sum of squares rule to give the standard uncertainty in the volume of the solution:

$$u(v) = \sqrt{0.0577^2 + 0.0173^2 + 0.015^2} = 0.0621 \text{mL}$$

Molecular mass of dopamine hydrochloride

The molecular formula for dopamine hydrochloride (3-hydroxytyramine hydrochloride) is $C_8H_{11}NO_2.HCl$. The atomic weights and their associated uncertainties are obtained from IUPAC tables [36]. A rectangular distribution is assumed for the uncertainties quoted by IUPAC. The standard uncertainties were therefore obtained by dividing the quoted uncertainties by √3.

Element	**Atomic weight**	**Quoted uncertainty**	**Standard uncertainty**
C	12.0107	0.0008	0.00046
H	1.00794	0.00007	0.000040
N	14.0067	0.0002	0.00012
O	15.9994	0.0003	0.00017
Cl	35.453	0.002	0.0012

The calculation of the molecular mass and its associated standard uncertainty is shown below. The standard uncertainty in the molecular mass is calculated by combining the values in the last column using the square root of the sum of squares rule.

	Calculation of molecular mass	Value	Calculation of standard uncertainty	Value
C_8	8 x 12.0107	96.0856	8 x 0.00046	0.0037
H_{12}	12 x 1.00794	12.09528	12 x 0.000040	0.00048
N	1 x 14.0067	14.0067	1 x 0.00012	0.00012
O_2	2 x 15.9994	31.9988	2 x 0.00017	0.00034
Cl	1 x 35.453	35.453	1 x 0.0012	0.0012
	Molecular mass	*189.639*	*Standard uncertainty*	*0.00392*