DETERMINATION OF MEASUREMENT UNCERTAINTY IN THE ANALYSIS OF SODIUM LACTATE USING THE HPLC METHOD

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DECLARATION

I, Mrs Rehana Fakir declare that this research report is my own work. It is being submitted for the degree of Master of Science in Medicine in Pharmaceutical Affairs in the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination at this or any other University.

..... [Signature of candidate]

.....day of.....[month], 2007

ABSTRACT

Measurement Uncertainty is a parameter associated with the result of a measurement that defines the range of the values that could reasonably be attributed to the measured quantity. When uncertainty is evaluated and reported in a specified way, it indicates the level of confidence that the value actually lies within the range defined by the uncertainty interval. Uncertainty is an unavoidable part of any measurement and it becomes significant when results are to be close to a specified limit. A proper evaluation of uncertainty is good professional practice and can provide laboratories and customers with valuable information about the quality and reliability of a result.

Any measurement is subject to imperfections; some of these are due to random effects, such as short-term fluctuations in temperature, humidity and air-pressure or variability in the performance of the analyst. Repeated measurements will show variation because of these random effects. Other imperfections are due to the practical limits to which correction can be made for systematic effects, such as offset of a measuring instrument, drift in its characteristics between calibrations, personal bias in reading an analogue scale or the uncertainty of the value of a reference standard.

The uncertainty is a quantitative indication of the quality of the result. It gives an answer to the question, how well does the result represent the value of the quantity being measured? Many important decisions are based on the results of chemical quantitative analysis. The results are used, for example, to estimate yields, to check materials against specifications or statutory limits, or to estimate monetary value. Whenever decisions are based on analytical results, it is important to have some indication of the quality of the results, that is, the extent to which they can be relied on for the purpose in hand.

In this study, the measurand (Sodium Lactate Assay) is specified in terms of a formula. All uncertainty sources in the Analytical procedure are identified. The uncertainty components are then quantified using the International Organisation for Standardisation on the Guide to the Expression of Uncertainty in Measurement (ISO GUM) approach in order to ascertain the influence of the various uncertainty sources. The combined uncertainty is then calculated using statistical analysis.

In this study, the ISO GUM approach was used to determine measurement uncertainty in the analysis of sodium lactate using the HPLC method.

The results obtained showed that:

- The uncertainty originating from sample preparation was negligible.

- ❖ The uncertainty components from weighing and volume measuring were small.
- ❖ 71 % of the uncertainty contribution was from instrumental (HPLC) measurements

An uncertainty value of 0,0621 mg/ml was obtained for the determination of sodium lactate using the HPLC method.

This value can now be incorporated when reporting a sodium lactate result on a finished product containing sodium lactate in solution. For example, in this study, a value of 2,93 mg/ml was found. The final result may be reported as:

2,93 ± 0,0621 mg/ml, which means:

The 'true" content of sodium lactate in the solution is in the range 2,87 – 2,99 g/l with 95% probability (in the case of normal distribution).

These results are significant because often, a result is compared with a limiting value defined in a specification or regulation. In this case, knowledge of the uncertainty shows whether the result is well within the acceptable limits or only just makes it. Occasionally a result is so close to the limit that the risk associated with the possibility that the property that was measured may not fall within the limit, once the uncertainty has been allowed for, must be considered.

Background

The purpose of this report is to address the use of Measurement Uncertainty testing information

generated from this study for the determination of Sodium Lactate by High Performance Liquid Chromatography (HPLC). Currently, results are being reported by taking measurements and data with no degree of confidence. Sodium lactate is used as a source of sodium ions when a systemic alkalizing action is desirable. Sodium lactate has two advantages over sodium bicarbonate. First, it can withstand sterilisation temperatures. Second, its effect is achieved slowly and over a longer period of time since there is a time lag between administration and the appearance of its bicarbonate oxidation product. Sodium lactate is metabolised by the liver to equivalent bicarbonate.

Results and Discussion

An uncertainty estimation procedure has been developed and applied to uncertainty calculations with actual experimental data. The contribution of the various uncertainty components is discussed.

The uncertainty originating from sample preparation is considered negligible. The uncertainty components from weighing and volume measuring were small. The uncertainty contribution from instrumental measurements was much higher. The effects of cross-contamination and dissolved oxygen are discussed. In addition, the effects of non-linearity are also discussed.

Conclusion

An ISO GUM measurement uncertainty estimation procedure was developed for a liquid-chromatographic drug quality control method – Assay of Sodium Lactate in Sodium Lactate solutions. Detailed analysis of contributions of the various uncertainty sources was carried out. The results were calculated based on the definition of the measurand, and it was demonstrated that unequivocal definition of the measurand is essential in order to get a rigorous uncertainty estimate.

Recommendation

For solutions containing sodium lactate, the analytical method should be revised to incorporate the value determined for uncertainty. Results should be reported as a range showing the 95 % confidence interval. This recommendation and approach is consistent with ISO GUM principles, and the recognition that test data generated according to this study can be used to support this recommendation.

SYNOPSIS

1. Specify the measurand

The quantity of Sodium Lactate was determined by HPLC. An In-house procedure was used. This procedure included all the factors that could significantly affect the measurement result including sampling procedures, sample preparation procedures as well as the test or calibration or calibration method used.
An equation for the analyte was formulated by expressing mathematically the relationship between the measurand and all of the input quantities upon which the measurand depends. This mathematical model contained every quantity that could contribute significantly to the uncertainty of the result of the measurement.
Variability was considered negligible as the analyte was measured in a homogeneous solution.

2. Identifying the uncertainty sources

A comprehensive list of relevant sources of uncertainty was assembled. In forming the required list of uncertainty sources it is usually convenient to start with the basic expression used to calculate the measurand from intermediate values. All the parameters in this expression had an uncertainty associated with its value and were therefore potential uncertainty sources. In addition there were other parameters that did not appear explicitly in the expression used to calculate the value of the measurand, but which nevertheless affected the measurement results, eg. Temperature

3. Quantifying the uncertainty sources

The value of each uncertainty source was estimated either by statistical analysis of repeated observations or by taking a value from a calibration certificate or by estimating the uncertainty based on Type A or Type B statistical observations of data.

For type A assessments it is necessary to choose the appropriate statistical method. For the situation where the average of several measurements is taken, there is an established method, which yields a standard uncertainty that is a measure of the dispersion of the measured value. The procedure is to calculate the mean of the set of readings and a standard deviation known as the *Experimental Standard Deviation of the Mean.* When data is treated to find a "best fit" such as when a curve is fitted, the standard techniques will also yield a standard uncertainty. Type A assessments are perhaps the most straightforward to make.
For Type B assessments the knowledge and understanding of the method is crucial. Each component must be examined to determine its limits, that is the range of dispersion, and the nature of the dispersion. This means that it must be decided if the uncertainty component is expected to cluster near a central value or is evenly dispersed across its range, or if it is more likely to be at the extremes. This analysis is called determining the distribution curve of the uncertainty. Once the range and nature of the dispersion have been decided, specific formulas are applied to find the standard uncertainty component.

4. Evaluating the standard uncertainty of each uncertainty source

The standard uncertainty of the estimates of the values for the uncertainty sources were evaluated either based on Type A or Type B observations.

5. Calculating the measurement result

The result of the measurement based on the mathematical model adopted and the estimates of the input quantities were calculated.

6. The Combine standard uncertainty

The combined standard uncertainty of the measurement result from the standard uncertainties and covariances of the input estimates was determined.

7. The expanded uncertainty

The combined standard uncertainty was multiplied by a coverage factor of 2 (95% confidence interval) to provide an expanded uncertainty.

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Table of Contents

Table of Contents (cont.)

List of Figures

List of Tables

List of Abbreviations

GUM: Guide to the Expression of Uncertainty in Measurement.

ISO: International Organisation for Standardisation.

HPLC: High Performance Liquid Chromatography.

CRM: Certified Reference Material

DCHA: Dicyclohexylamine

PDA: Photodiode Array

CoA: Certificate of Analysis

ESDM: Estimated Standard Deviation of the Mean

AU: Absorbance Unit

1.0 Introduction

Uncertainty is one of the most important quality characteristics of any measurement. Uncertainty estimation is becoming a standard requirement in chemical measurement. During the last few years progress has been made in the development of methodology for the uncertainty estimation in chemical measurement. Still, due to peculiarities of chemical measurement, more work is required before an ideal situation is achieved in this field. This is explicitly demonstrated by interlaboratory comparison measurements, where the differences between the results of different participants are often many times larger than the uncertainty estimates declared by the participants. Often, a result is compared with a limiting value defined in a specification or regulation. In this case, knowledge of the uncertainty shows whether the result is well within the acceptable limits or only just makes it.

Occasionally a result is so close to the limit that the risk associated with the possibility that the property that was measured may not fall within the limit, once the uncertainty has been allowed for, must be considered.

The complexity of uncertainty estimation in chemical measurements as compared to the physical ones has several origins:

(a) chemical measurements are more difficult to model,
(b) there are more uncertainty sources, that
(c) are often very difficult to quantify,
(d) inhomogeneity of the analysis objects and most importantly
(e) serious problems related to selectivity of measurement and separation of the chemical species to be determined from the analysis objects

Uncertainty sources in liquid chromatographic analysis have been extensively studied and a lot has been published on this subject. In papers on liquid chromatographic methods the accuracy of the result is often discussed, but usually not expressed as combined uncertainty that accounts for all the uncertainty sources. Instead, other accuracy criteria (e.g. day-to-day repeatability, root mean square deviations from the calibration graph, etc.) are mostly used.

Many published studies are based on the Guide to the Expression of Uncertainty in Measurement (GUM) method by the International Organisation for Standardisation (ISO). [1]

Other techniques for estimation of uncertainty are e.g. EURACHEM/CITAC GUIDE (2000) which is used in chemical applications and the IAEA TecDoc 1401 (2004) which is used for uncertainty measurements in nuclear applications. The A2LA Guide for the Estimation of Measurement Uncertainty is used for physical measurements e.g. tensile strength.

The GUM Workbench programme is available which can be used to simplify the calculations of standard uncertainties.

The ISO method involves the following steps:

1. Define the measurand
2. Build the model equation
3. Identify the sources of uncertainty
4. Evaluate the sources of uncertainty and calculate the value of the result
5. Estimate the standard uncertainty of uncertainty sources.
6. Calculate the combined standard uncertainty of the result
7. Present the result (as expanded uncertainty)
8. Report the measurement result and associated uncertainty estimate. [2]

Step 1: Specify the measurand

Express the mathematical relationship between the measurand Y and the input quantities x_i: [2]

$Y = f(x_i, \ldots x_n)$.

Step 2: Derive the mathematical model

Express mathematically the relationship between the measurand and all of the input quantities upon which the measurand depends. This mathematical model should contain every quantity, including all corrections, that can contribute significantly to the uncertainty of the result of the measurement. [2]

Step 3: Quantify the influence quantities

Estimate the value of each input quantity either by the statistical analysis of repeated observations or by other means such as taking the uncertainty of a reference standard from a calibration certificate, estimating temperature effects on test or calibration results based on theoretical predictions, estimating the uncertainty of physical constants based on data in reference books and so on. [2]

The measurement process must be well defined and under control before any assessment of uncertainties is attempted. When this has been achieved, if sufficient resolution is available, it will be found that repeated measurements still yield different values.

It will be found that the measured values vary randomly, often without apparent cause, hence the term ***Random Errors***. The values will cluster around a central value. The best estimate of this central value is the mean or average of the measured values, but the mean may not be the true value. It may be biased away from the true value by a combination of errors that are essentially constant.

Whilst precise determination is frequently not possible, there will usually be extreme limits, which can be estimated or measured which will encompass the possible value of such errors. These errors are called ***Systematic Errors***. Repeated measurements will not reduce the size of a systematic error or, in general, improve the knowledge of its magnitude or sign.[3]

Random Errors

The random variations in the results of measurements are called random errors. Sources of these errors are many and are inherent in the measuring instrument, the item under test, the test procedure and the test environment. Sources of random errors include varying influence parameters which cannot be adequately controlled, such as air currents and ambient temperature fluctuations, relative humidity variations, power source disturbances, mechanical vibrations from machinery.[3]

The estimation of the random components may be done in the manner described later using a type A analysis. This requires that the measurements be repeated a number of

times. A simple manipulation of the data produces a mean value and a standard deviation.[3]

Systematic Errors

Systematic errors may be considered to be corrections that are not applied because their actual magnitude and sign are not precisely known. It is a characteristic of systematic errors that they can neither be reduced, nor our knowledge of their magnitude improved, by taking the average of repeated measurements under constant conditions.[3]

Step 4: Evaluate the standard uncertainty of each influence quantity

Evaluate the standard uncertainty of the estimates of the values for the input quantities. For the uncertainty of an input estimate based on the statistical analysis of repeated observations, the estimate is made as described below (Type A). For estimates obtained by other means, the standard uncertainty is evaluated as a Type B as described below. [2]

Type A assessment

A type A evaluation is an evaluation of a standard uncertainty by statistical calculations. This is only possible when a series of observations such as repeated measurements are feasible. The most common reason for taking repeated measurements is to minimize the effect of random errors or noise. [3]

Type B evaluation of uncertainty components

Type B determination of uncertainties is determination of uncertainties by means other than by established statistical methods. It involves using data provided by others, such as uncertainties on a calibration certificate or from the manufacturer's specifications, reference tables and books. In many instances, Type B evaluation involves estimation of systematic errors. For many systematic errors the magnitude of

the error is not known, only the limits within which it lies. The next step involves making a decision about the distribution that describes the dispersion of the errors. [3]

Rectangular distribution

It may often be assumed that the errors have equal probability of having a value anywhere in that range and, unlike random errors, are not more likely to be near the mean value than the extremes. This is a rectangular distribution, which is illustrated in Fig 1. The maximum and minimum limits are assumed to be equal and often are denoted by ***a***, which is called the semi-range. [3]

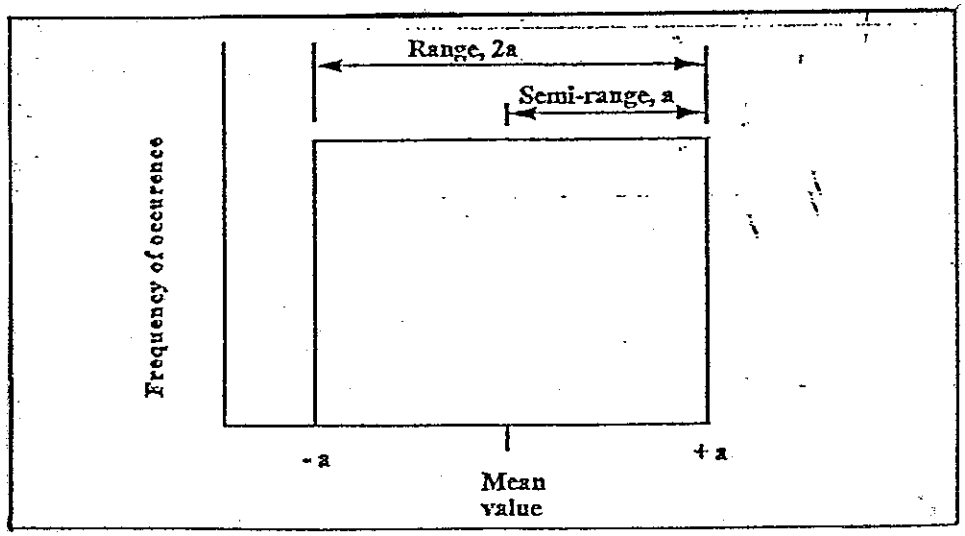

Fig 1. A rectangular distribution with semi-range ***a***

Such a distribution is capable of mathematical analysis and it can be shown that the standard deviation and hence the standard uncertainty, $u(x_i)$, is given by:

$$u(x_i) = \frac{a}{\sqrt{3}}$$

where ***a*** is the semi-range

Type B evaluations are applicable to all types of distributions. The rectangular distribution may be considered the distribution of minimal knowledge. This is because all we know are the limits and, in the absence of further knowledge, assume That any value is equally probable.

Triangular distribution

Another commonly used distribution is the triangular distribution. The limits may be estimated in the same way as for rectangular distribution. It is used when there is evidence that the values near the mean are the most probable and, as the limits are approached, the probability decreases to zero. It represents less knowledge than in the normal distribution case, but more knowledge than the rectangular distribution case. For a triangular distribution, the uncertainty is calculated by: [3]

$$u(x_i) = \frac{a}{\sqrt{6}}$$

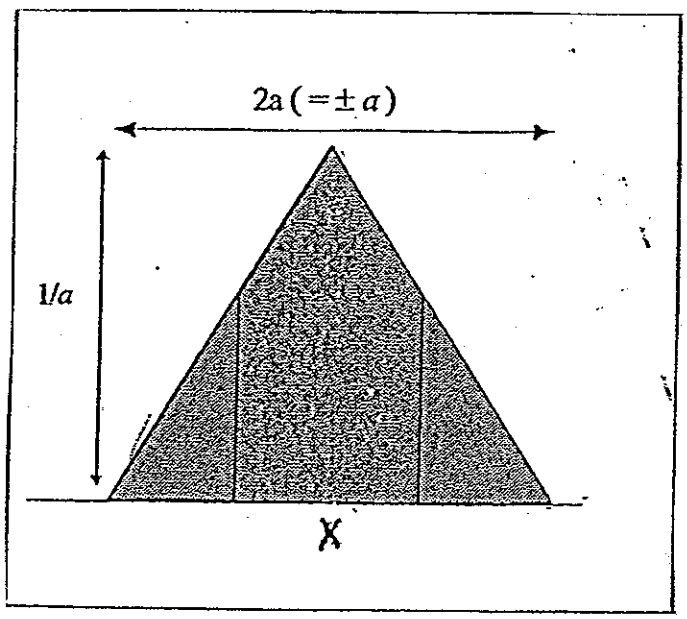

Fig 2. A triangular distribution with semi-range *a*

Step 5: Estimate the standard uncertainty of uncertainty sources.

Calculate the result of the measurement according to the equation from Step 1: $Y = f(x_i, \ldots x_n)$.

Step 6: Calculate the combined standard uncertainty of the result

Determine the combined standard uncertainty of the measurement result from the standard uncertainties and covariances of the input estimates. [2]

Note: Steps 5 and 6 can be done with software such as GUM Workbench

Step 7: Determine the expanded uncertainty.

Multiply the combined standard uncertainty by the coverage factor associated with the desired level of confidence. [2]
If necessary, calculate an expanded uncertainty U, [y ± U] by multiplying the standard uncertainty $u_c(y)$ with an appropriate coverage factor k: $U = k. u_c(y)$. For a 95% confidence interval, $k \approx 2$

Step 8: Report the measurement result and associated uncertainty estimate.

Report the measurement result and either the combined standard uncertainty or the expanded uncertainty. [2]

Also other methods have been used for uncertainty estimation employing validation data, robustness tests and interlaboratory comparisons.
Uncertainty estimation via the ISO GUM – an approach based on modelling and detailed study of the measurement and evaluating the magnitude of all the significant uncertainty contributions- gives not only the uncertainty estimate characterising the quality of the result but also the uncertainty budget displaying the relative contributions of the uncertainty sources. The uncertainty budget is a powerful tool for optimising the analytical method as it allows one to see where to focus the efforts, and its availability is a distinct advantage over other uncertainty estimation methods. However, this advantage is realised only when the measurement procedure is analysed in a detailed way, with all the important uncertainty components having been taken into account and adequately characterised. [5]

Measurement uncertainty is useful:

- To estimate the reliability of the measurement result
- To be able to compare two measurement results
- To establish traceability
- With uncertainty analysis one has to thoroughly analyze the method
- Information is obtained for improving the method. [4]

An example of the way a result is reported using measurement uncertainty principles is:

C svt = 9,67 mg/tab ± 0,23 mg/tab (k= 2), norm

The "true" content of simvastatin in the tablet is in the range of 9,44 – 9,90 mg/tab with a probability of 95 % (in the case of normal distribution).

1.1 Definition of Uncertainty

It is a parameter, associated with the result of a measurement that defines the range of the values that could reasonably be attributed to the measured quantity.[5]

When uncertainty is evaluated and reported in a specified way it indicates the level of confidence that the true value actually lies within the range defined by the uncertainty interval.

Any measurement is subject to imperfections; some of these are due to random effects, such as short-term fluctuations in temperature, humidity and air-pressure or variability in the performance of the measurer. Repeated measurements will show variation because of these random effects. Other imperfections are due to the practical limits to which correction can be made for systematic effects, such as offset of a measuring instrument, drift in its characteristics between calibrations, personal bias in reading an analogue scale or the uncertainty of the value of a reference standard.

The uncertainty is a quantitative indication of the quality of the result. It gives an answer to the question, how well does the result represent the value of the quantity being measured? It allows users of the result to assess its reliability, for example for the purposes of comparison of results from different sources or with reference values. Confidence in the comparability of results can help to reduce barriers to trade. [6]

1.2 Problem Statement

One of the problematic areas that the QC laboratory must address is the inconsistencies of the Sodium Lactate results obtained on the finished product using the HPLC method. Although validated methods are used, results are often borderline and this leads to much doubt when release of product into market is required. Batches are known to have been dumped at enormous costs to the company. These decisions are not based on sufficient available data and the risk of encountering Type I and Type II errors are extremely high.

1.3 The Importance of Sodium Lactate

Peritoneal dialysis is the most common form of home dialysis and has become an alternative method of treatment for patients with end-stage renal disease.
One of the systemic abnormalities that renal replacement therapy must address is the metabolic acidosis that occurs with progressive renal insufficiency. This acidosis reflects the inability of the failing kidney to excrete the endogenous acid generated by normal metabolism. The major factors contributing to endogenous acid production include oxidation of sulphur-containing amino acids (methionine, cystine, cysteine), renal or gastric excretion of metabolizable organic anioins, metabolism of phosphoproteins, and oxidation of organic cations (eg. Lysine, arginine, histidine, etc). If uncontrolled, chronic metabolic acidosis leads to a variety of skeletal and metabolic abnormalities. These include growth retardation in children, osteopenia , and impaired protein metabolism.
Furthemore, underlying renal osteodystrophy is likely to be worsened by the acidosis. In children, chronic acidosis may be associated with symptoms such as anorexia, nausea and muscle weakness. Hence, management of acidosis is an integral part of the overall management of dialysis patients. [7]

In the clinical practice of peritoneal dialysis, regeneration of depleted buffer stores is accomplished by including a buffer in the dialysate in concentrations greater than those in plasma. This buffer then enters the blood by diffusive mass transfer across the peritoneal membrane. In initial applications of peritoneal dialysis, bicarbonate was used as the buffer –repletion agent . However, problems with calcium carbonate

precipitation during sterilisation and long-term storage resulted in the replacement of bicarbonate by ***lactate.*** Current peritoneal dialysis solutions contain 35 to 40 mEq/L lactate. Clinical experience suggests that lactate based solutions are adequate in maintaining acid-base balance in peritoneal dialysis patients.[7]

Lactate used in peritoneal dialysis solutions is obtained by either fermentation of sugars or by chemical synthesis. Lactic acid prepared by fermentation of sugars is L(+) isomer of lactate, while that manufactured synthetically is an equal mixture of L(+) and D(-) sterioisomers (racemic lactate). The L(+) isomer is the natural form of lactate in all mammals. During the early years of peritoneal dialysis, the only available commercial source of pharmaceutical quality lactate was that prepared by the synthetic route. Presently, pharmaceutical quality L(+) lactate prepared by fermentation of sugars is available and accordingly present-day peritoneal dialysis solutions contain either racemic or L(+) lactate as the buffer to correct metabolic acidosis in dialysis patients. Since the L(+) isomer is the natural form in humans, one could argue, at least on theoretical grounds, its preference over the racemic mixture. [7]

1.4 Metabolism of Lactate

In order for lactate to be a clinically useful buffer, it must be metabolised at a rate that is fast enough so as not to produce lactic acidosis. L(+) lactate is the form normally found in mammalian species and its metabolism in humans is well understood. L(+) lactate in man is readily converted to pyruvate by L(+) lactate dehydrogenase. Pyruvate may enter a number of metabolic pathways leading to electroneural end products. These pathways include oxidation to carbon dioxide and water through the Krebs cycle, conversion to glucose and glycogen via the Embden-Myerhof pathway, and conversion to lipids. The liver is the major site of overall lactate metabolism, with over 50% occurring in this organ. The kidneys account for an additional 30% of lactate metabolism, with skeletal muscle and heart accounting for the remainder . In normal subjects, the total body clearance of L(+) lactate was estimated to be 15.2 ml/kg/min.[7]

1.5 Aim of Study

The aim of the study is to evaluate and report the uncertainty value of sodium lactate in solutions containing sodium lactate, using the HPLC method, in such a way that it indicates the level of confidence that the true value actually lies within the range defined by the uncertainty interval.

1.6 Objective

The objective of the study is to be able to perform an uncertainty estimation using the ISO GUM method -- an approach based on modelling and detailed study of the measurement and evaluating the magnitude of all the significant uncertainty contributions.
Furthermore, it is intended to provide recommendations to the Quality Assurance Laboratory that the analytical result for sodium lactate in sodium lactate solutions should be reported by incorporating the uncertainty value determined in this study and reporting the result as a range showing the 95% confidence interval.

2.0 Materials and Methods

The study will identify as many sources of uncertainty as possible and account for them by appropriate precision and trueness studies. Any additional sources of uncertainty can evaluated by other means such as calibration certificates, published data, etc. It may not be necessary to evaluate every source of uncertainty if they are deemed insignificant, unless there are a large number of them. Uncertainty components that are less than one third of the largest component need not be evaluated in detail.

A preliminary estimate of the contribution of each component or combination of components to the uncertainty was made and those factors that were not significant were eliminated. The uncertainty contributions were expressed as standard deviations, and combined according to the appropriate rules, to give a combined standard uncertainty. The appropriate coverage factor (usually 2) should be applied to give an

expanded uncertainty. The uncertainties can be shown for convenience on an Ishikawa (cause and effect) diagram as illustrated in Fig 3.

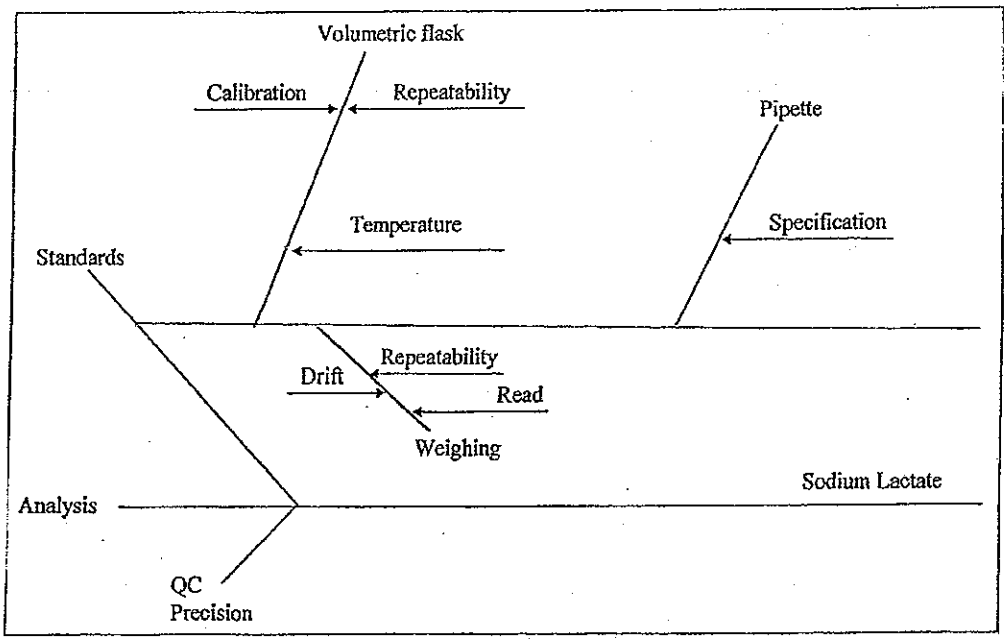

Figure 3. An Ishikawa (cause and effect) diagram of uncertainty sources

2.1 Experimental

2.1.1 Chemicals, solutions and consumables

The following chemicals were used for preparation of the eluent:

Formic Acid: ≥ 88 % ACS Reagent grade, or equivalent

Dicyclohexylamine (DCHA) : 99 % purity

Reference Standard:

A USP Sodium Lactate Reference Standard was used (Lot JOE 249 Purity100 %)

Other reagents:

Sodium Acetate Anhydrous: Reagent grade

Methanol: HPLC grade

Purified water was prepared in-house using the Milli-Q water purification system

Preparation of 2 litres of Mobile Phase:

Using volumetric pipettes, add 2,0 ml of Formic acid and 2,0 ml of DCHA to about

1 500 ml distilled water. Stir on a magnetic stirrer for approximately 10 minutes. Quantitatively transfer to a 2 litre volumetric flask and dilute to volume with water. Mix well. Filter and degas (5 minutes).

System Suitability Preparation:

Accurately weigh 0,15 g of Sodium Acetate Anhydrous and 0,15 g of Sodium Lactate and quantitatively transfer to a 50 ml volumetric flask. Dissolve and dilute to volume with distilled water.

Standard Preparation:

Standard 1: Accurately weigh 125 mg into a 25 ml volumetric flask. This the stock standard from which the other two standards were made up.
Standard 2: 6 ml of Standard 1 was pipetted into a 10 ml volumetric flask and diluted to volume with water.
Standard 3: 4 ml of Standard 1 was pipetted into a 10 ml volumetric flask and diluted to volume with water.

Sample Solution Preparation:

Preparation was not required as the sample is a homogeneous liquid and may be assayed directly using the appropriate working standard concentration range.

2.1.2 Apparatus and chromatographic conditions

A Waters 2690 Separation Module with Waters 996 photodiode array (PDA) detector controlled by Empower 2 Software was used for the quantitative analysis.
All the weightings were carried out using analytical balance RDI 0503 (Mettler Toledo AG 204) with a Total uncertainty of measurement of +/- 0.0005 g
Quantitative analyses were carried out on Discovery C18 04/101/PMc 10 x 4,6 mm analytical column. Temperature was 20 °C ± 4 °C and injection volume 20,00 µl. Autosampler was used for injection. The eluent flow rate was 1,0 ml/min. The analytical wavelength was 210 nm.

2.1.3 Preparation of standard solutions:

$C_3 = m_3 * Pstd / (V_{3\_25} * 100)$

Note: Due to the limited amount of CRS standard available, only mass for C3 was weighed out and treated as the Stock solution which is also part of the calibration standards. The other two standards C1 and C2 were prepared by taking dilutions from the stock standard C3. Hence the standards were prepared as follows:

C3 : weighed 125,8 mg of CRS standard into a 25 ml volumetric flask.

$$C3 = \frac{125{,}8 \times 100}{25 \times 100} = 5{,}032 \text{ mg/ml}$$

C2: 6,0 ml of C3 was pipetted into a 10 ml volumetric flask and diluted with distilled water.

C2 = 5,032 x 6/10 = 3,0192 mg/ml

C1: 4,0 ml of C3 was pipetted into a 10 ml volumetric flask &diluted to volume.

C1 = 5,032 x 4/10 = 2,0128 mg/ml

2.2 Table 1: List of Uncertainty sources

Quantity	Unit	Definition
A_1	AU	Peak area of the 1. calibration standard solution
A_{1drift}	AU	Drift uncertainty component of the peak area of the 1. calibration standard solution
$A_{1integr}$	AU	Integration uncertainty component of the peak area of the 1. calibration standard solution.
A_{1rep}	AU	Value and the repeatability uncertainty component of the peak area of the 1. calibration standard solution.
A_2	AU	Peak area of the 2. calibration standard solution
A_{2drift}	AU	Drift uncertainty component of the peak area of the 2.

		calibration standard solution
$A_{2integr}$	AU	Integration uncertainty component of the peak area of the 2. calibration standard solution.

Table 1: List of Uncertainty sources (cont.)

A_{2rep}	AU	Value and the repeatability uncertainty component of the peak area of the 2. calibration standard solution.
A_3	AU	Peak area of the 3. calibration standard solution
A_{3drift}	AU	Drift uncertainty component of the peak area of the 3. calibration standard solution
$A_{3integr}$	AU	Integration uncertainty component of the peak area of the 3. calibration standard solution.
A_{3rep}	AU	Value and the repeatability uncertainty component of the peak area of the 3. calibration standard solution
A_{sample}	AU	Peak area of the sample solution
A_{sample_drift}	AU	Drift uncertainty component of the sample peak area
A_{sample_integr}	AU	Integration uncertainty component of the sample peak area
A_{sample_nonlin}	AU	Uncertainty component of the sample peak area that takes into account the slight non-linearity of the calibration graph
A_{sample_rep}	AU	The value of the sample solution peak area together with its repeatability uncertainty.
AvgA	AU	Interim quantity for regression statistics calculation
AvgC	mg/ml	Interim quantity for regression statistics calculation
b_0	AU	Intercept of the calibration line
b_1	AU*ml/mg	Slope of the calibration line
C_1	mg/ml	Concentration of the 1. calibration standard solution
C_2	mg/ml	Concentration of the 2. calibration standard solution
C_3	mg/ml	Concentration of the 3. calibration standard solution
m_3	mg	Mass of the Sodium Lactate CRS taken for preparation of the 3. calibration standard solution (stock solution)

m_{3_drift}	mg	Drift component of uncertainty of mass m_3
m_{3_rep}	mg	Value of the mass of the Sodium Lactate CRS taken for preparation of the 3. calibration standard solution together with repeatability component of uncertainty.

Table 1: List of Uncertainty sources (cont.)

m_{3_read}	mg	Readability component of uncertainty of mass m3
n	unitless	Number of points on the calibration line n=3
P_{std}	%	Purity of the Sodium Lactate CRS
V_{1_10}	ml	Volume of the 1. calibration standard solution
$V_{1\_10\_cal}$	ml	Value of the volume of the 1. calibration standard solution together with calibration component of uncertainty
$V_{1\_10\_repeat}$	ml	Repeatability component of uncertainty of volume V_{1_10}
$V_{1\_10\_temp}$	ml	Uncertainty component of volume V_{1_10} due to temperature difference from 20 °C
V_{2_10}	ml	Volume of the 2. calibration standard solution
$V_{2\_10\_cal}$	ml	Value of the volume of the 2. calibration standard solution together with calibration component of uncertainty
$V_{2\_10\_repeat}$	ml	Repeatability component of uncertainty of volume V_{2_10}
$V_{2\_10\_temp}$	ml	Uncertainty component of volume V_{2_10} due to temperature difference from 20 °C
V_{3_25}	ml	Volume of the 3. calibration standard solution
$V_{3\_25\_cal}$	ml	Value of the volume of the 3. calibration standard solution together with calibration component of uncertainty
$V_{3\_25\_repeat}$	ml	Repeatability component of uncertainty of volume V_{3_25}
$V_{3\_25\_temp}$	ml	Uncertainty component of volume V_{3_25} due to temperature difference from 20 °C
V_{1_4}	ml	Volume of the 4 ml pipette
$V_{1\_10\_cal}$	ml	Value of the volume of the 4 ml pipette together with calibration component of uncertainty
$V_{1\_10\_repeat}$	ml	Repeatability component of uncertainty of volume V_{1_4}
$V_{1\_10\_temp}$	ml	Uncertainty component of volume V_{1_4} due to temperature difference from 20 °C

Table 1: List of Uncertainty sources (cont.)

$V_{1\_2\ temp}$	ml	Uncertainty component of volume V_{1_2} due to temperature difference from 20 °C
Δt	°C	Difference of solution temperature from 20 °C
γ_w	1/°C	Thermal expansion coefficient of water
ΣAC	AU*mg/ml	Interim quantity for regression statistics
ΣCC	mg^2/ml^2	Interim quantity for regression statistics

3.0 RESULTS

The Sodium Lactate concentration is given by the following model equation:

$$[\text{Na L}]\ g/l = \frac{A_{samp} \times m_{stnd} \times 1000 \times P}{A_{stnd} \times 100\ ml \times 100}$$

Where A_{samp} = Peak area of the sample solution

A_{stnd} = Peak area of the standard solution

m_{stnd} = mass of the standard in g

P_{stnd} = Purity of standard

V_{100} ml = 100 ml volumetric flsak

3.1 Uncertainty of Weighing

The following uncertainty sources are taken into account:

1. Repeatability of the balance
2. Drift of the balance
3. Readability of the display of the balance (the readability of the balance is ± 0,1%)

Table 2: List of Uncertainty Quantities for weighing

Quantity	Unit	Definition
m	g	Mass of the sample
m_{rep}	g	Value of the mass together with repeatability component of uncertainty
m_{drift}	g	Drift component of uncertainty of mass
m_{read}	g	Readability component of uncertainty of mass

Model Equation:

$m = m_{rep} + m_{drift} + m_{read}$

m: Result

3.1.1 m_{rep}: Type A summarized

Mean: 125,641 mg

Standard Uncertainty: 0,0213307 mg

From the weighing checks the standard deviation at 125 mg level was calculated at:
s = 0,09539364 mg (20 determinations)

Table 3: Data from repeatability experiment of weighing

125,604 mg	125,604 mg
125,711 mg	125,711 mg
125,683 mg	125,656 mg
125,697 mg	125,711 mg
125,682 mg	125,508 mg
125,797 mg	125,589 mg
125,712 mg	125,682 mg
125,589 mg	125,735 mg
125,397 mg	125,589 mg
125,490 mg	125,666 mg
Mean x = 125,641 mg s = 0,09539364	

$$ESDM = \frac{s}{\sqrt{n}} = \frac{0,09539364}{\sqrt{20}} = 0,0213307 \text{ mg}$$

This uncertainty estimate was obtained for the balance used. It refers to tared weighing (ie: it takes the taring operation into account), so the repeatability of taring need not be included separately.

3.1.2 m_{drift}: Type B rectangular distribution

Halfwidth of limits : 0,2 mg

Standard Uncertainty: $\frac{0,2}{\sqrt{3}} = 0,11547$ mg

This uncertainty estimate was obtained for the balance used. It refers to tared weighing (ie: it takes the taring operation into account), so the drift of taring need not be included separately.

3.1. m_{read}: Type B rectangular distribution

Halfwidth of limits : 0,25 mg

Standard Uncertainty: $\frac{0,25}{\sqrt{3}}$ = 0,144337 mg

The uncertainty component of rounding of the last digit was taken as half of the last digit i.e (half of 0,5 mg)

The uncertainty of the Mettler Toledo AG 204 analytical balance can be determined from the Microsep Calibration certificate which abates an uncertainty at the 125 mg level as:-

m_{read} = 125 ± 0,0005g [95% c.i.] from the Microsep certificate

= 125 ± 0,5 mg

The half of the last digit is calculated, resulting in

0,5/2

= 0,25 mg

Standard Uncertainty: $\frac{0,25}{\sqrt{3}}$ = 0,144337 mg

3.1.4 Combined uncertainty of standard weighing:

$U_{Weighing} = \sqrt{(0,0213307)^2 + (0,11547)^2 + (0,144337)^2}$

= 0,186068 mg

3.2 Uncertainty of Standard Purity used for Assay:

This contribution was evaluated based on the standard substance Certificate of Analysis (CoA). Since no information on the distribution of the purity was available from the certificate, rectangular distribution was assumed. (Type B)

3.2.1 Purity of Sodium Lactate standard: 100% ± 0.1%

1.0 ± 0.001

$$U_{Purity} = \frac{0.001}{\sqrt{3}} = 0{,}000577$$

3.3 Uncertainty of the 25 ml Volumetric Flask:

Due to a limited amount of Certified Reference Standard (CRS) material available, the stock standard solution was made up into a 25 ml volumetric flask which is also the third calibration standard.

The following components of uncertainty are taken into account:

1. Tolerance of the volume as specified by the manufacturer (Type B)
2. Uncertainty due to the filling repeatability (Type A)
3. Uncertainty of the volume due to temperature difference from 20 °C

Model Equation:

3.3.1 Volume of the 25 ml volumetric flask

Table 4: List of Uncertainty Quantities for 25 ml volumetric flask.

Quantity	Unit	Definition
V_{3_25}	ml	Volume of the 25 ml flask
$V_{3\_25\_cal}$	ml	Value of the volume together with calibration uncertainty contribution

$V_{3\_25\_repeat}$	ml	Uncertainty contribution of the volume due to repeatability
$V_{3\_25\_temp}$	ml	Uncertainty contribution of the volume due to temperature difference from 20 °C
γ	1/ °C	Thermal expansion coefficient of water
Δt	°C	Difference of liquid temperature from 20 °C

The volume is expressed as sum of three components. The V_{cal} carries the value together with calibration uncertainty.
The two other components carry only uncertainties, their values are set to zero.

$$V_{3\_25} = V_{3\_25\_cal} + V_{3\_25\_repeat} + V_{3\_25\_temp}$$

Uncertainty of the flask volume due to temperature difference from 20 °C

$$V_{temp} = V * \gamma * \Delta t$$

V_{3_25}: Result

3.3.2 $V_{3\_25\_cal}$ Type B – rectangular distribution
Value: 25.0 ml
Limits: ± 0,015 ml

$$U_{cal} = \frac{0,015}{\sqrt{3}} = 0,00866$$

3.3.3 $V_{3\_25\_repeat}$ Type B rectangular distribution
Value: 0 ml
Limits: 0,03 ml

The uncertainty of ± 0,03 ml corresponds to the uncertainty of plus minus 1 drop. With 25 ml volumetric flask this is a reasonable filling uncertainty estimate.

$$U_{repeat} = \frac{0,03}{\sqrt{3}} = 0.0173 \text{ ml}$$

3.3.4 $V_{3\_25\_temp}$ Interim result

γ: Constant
Value: 0,00021 1/°C

Δt: Type B rectangular distribution
Value: 0 °C
Limits: 3 °C

It is reasonable to assume that the temperature in the lab does not differ from 20 °C by more than 3 degrees.

$V_{3\_25\_temp} = V * \gamma * \Delta t$

$= 25 * 0,00021 * 3$
$= 0.01575$ ml

The standard uncertainty is calculated using the assumption of a rectangular distribution for the temperature variation i.e.

$$U_{Temp} = \frac{0.01575 \text{ ml}}{\sqrt{3}} = 0.0091 \text{ ml}$$

3.3.5 Combined Uncertainty of 25 ml volumetric flask using worst case:

$$U_{Volumetric} = \sqrt{(U_{cal})^2 + (U_{Repeat})^2 + (U_{Temp})^2}$$

$$= \sqrt{(0,00866)^2 + (0.0173)^2 + (0.0091)^2}$$

$$= 0,02138$$

3.4 Uncertainty of the 10 ml Volumetric Flask:

Due to a limited amount of Certified Reference Standard (CRS) material available, calibration standards C1 and C2 were made up into 10 ml volumetric flasks by diluting from stock standard.

The following components of uncertainty are taken into account:

1. Tolerance of the volume as specified by the manufacturer (Type B)
2. Uncertainty due to the filling repeatability (Type A)
3. Uncertainty of the volume due to temperature difference from 20 °C

Model Equation:

3.4.1 Volume of the 10 ml flask

Table 5: List of Uncertainty Quantities of the 10 ml volumetric flask

Quantity	Unit	Definition
V_{1_10}	ml	Volume of the 10 ml flask
$V_{1\_10\_cal}$	ml	Value of the volume together with calibration uncertainty contribution
$V_{1\_10\_repeat}$	ml	Uncertainty contribution of the volume due to repeatability
$V_{1\_10\_temp}$	ml	Uncertainty contribution of the volume due to temperature difference from 20 °C
γ	1/ °C	Thermal expansion coefficient of water
Δt	°C	Difference of liquid temperature from 20 °C

The volume is expressed as sum of three components. The V_{cal} carries the value together with calibration uncertainty.
The two other components carry only uncertainties, their values are set to zero.

$$V_{1\_10} = V_{1\_10\_cal} + V_{1\_10\_repeat} + V_{1\_10\_temp}$$

Uncertainty of the flask volume due to temperature difference from 20 °C

$V_{temp} = V * \gamma * \Delta t$

V_{1_10}: Result

3.4.2 $V_{1\_10\_cal}$ Type B – rectangular distribution
Value: 10.0 ml
Limits: ± 0,04 ml

$U_{cal} = \frac{0,04}{\sqrt{3}} = 0,023094$ ml

3.4.3 $V_{1\_10\_repeat}$ Type B rectangular distribution
Value: 0 ml
Limits: 0,03 ml

The uncertainty of ± 0, 03 ml corresponds to the uncertainty of plus minus 1 drop. With 10 ml volumetric flask this is a reasonable filling uncertainty estimate.

$U_{repeat} = \frac{0,03}{\sqrt{3}} = 0,0173$ ml

3.4.4 $V_{3\_25\_temp}$ Interim result

γ: Constant
Value: 0,00021 1/°C

Δt: Type B rectangular distribution
Value: 0 °C
Limits: 3 °C

It is reasonable to assume that the temperature in the lab does not differ from 20 °C by more than 3 degrees.

$V_{1\_10\_temp} = V * \gamma * \Delta t$

$= 10 * 0{,}00021 * 3$

$= 0.0063$ ml

The standard uncertainty is calculated using the assumption of a rectangular distribution for the temperature variation i.e.

$$U_{Temp} = \frac{0.0063 \text{ ml}}{\sqrt{3}} = 0.003637 \text{ ml}$$

3.4.5 Combined Uncertainty of 10 ml volumetric flask using worst case:

$$U_{Volumetric} = \sqrt{(U_{cal})^2 + (U_{Repeat})^2 + (U_{Temp})^2}$$

$$= \sqrt{(0{,}023094)^2 + (0.0173)^2 + (0.003637)^2}$$

$$= 0{,}0291$$

3.5 Uncertainty of 4 ml pipette

The following components of uncertainty are taken into account:

1. Tolerance or calibration of the volume as specified by the manufacturer
2. Uncertainty due to the repeatability of pipetting
3. Uncertainty of the volume due to temperature difference from 20 °C

The calculation assumes that the pipette has been thoroughly cleaned, and that no droplets are formed on the inner surface of the pipette after draining the liquid.

Model Equation:

3.5.1 Volume of the 4 ml pipette

Table 6: List of Uncertainty Quantities for the 4 ml pipette:

Quantity	Unit	Definition
V_{1_4}	ml	Volume of the 4 ml pipette
$V_{1\_4\_cal}$	ml	Value of the volume together with calibration uncertainty contribution
$V_{1\_4\_repeat}$	ml	Uncertainty contribution of the volume due to repeatability
$V_{1\_4\_temp}$	ml	Uncertainty contribution of the volume due to temperature difference from 20 °C
γ	1/ °C	Thermal expansion coefficient of water
Δt	°C	Difference of liquid temperature from 20 °C

The volume is expressed as sum of three components. The V_{tol} carries the value together with calibration uncertainty.
The other two components carry only uncertainties, their values are set to zero.

$$V = V_{tol} + V_{repeat} + V_{temp}$$

Uncertainty of the pipette volume due to temperature difference from 20 °C

$$V_{1\_4\_temp} = V * \gamma * \Delta t$$

V_{1_4}: Result

3.5.2 $V_{1\_4\_cal}$: Type B rectangular distribution

Value: 4.00 ml

Limits: ± 0.015 ml

U $_{cal}$: $\mathbf{U}_{cal} = \frac{0,015}{\sqrt{3}} = 0,00866$

3.5.3 $V_{1\_4\_repeat}$: Type A summarised

Mean: 0 ml

Standard Uncertainty: 0.016 ml

It may be estimated here that the repeatability standard uncertainty of pipetting is 0.4% of the pipette volume. This value may be assumed as a safe estimate.

U $_{repeat}$: $\mathbf{U}_{repeatl} = \frac{0,016}{\sqrt{3}} = 0,0092376$

3.5.4 $V_{1\_4\_temp}$ Interim result

γ: Constant

Value: 0,00021 1/°C

Δt: Type B rectangular distribution

Value: 0 °C

Limits: 3 °C

It is reasonable to assume that the temperature in the lab does not differ from 20 °C by more than 3 degrees.

$V_{1\_4\_temp} = V * \gamma * \Delta t$

$= 4 \times 0.00021 \times 3$ °C

$= 0.00252$ ml

$$U_{Temp} = \frac{0.00252}{\sqrt{3}}$$

$= 0.0014549$

3.5.5 Combined Uncertainty of 4 ml volumetric pipette using worst case:

$$U_{Volumetric} = \sqrt{(U_{cal})^2 + (U_{Repeat})^2 + (U_{Temp})^2}$$

$$= \sqrt{(0{,}00866)^2 + (0.0092376)^2 + (0.0014549)^2}$$

$= 0{,}0127$

3.6 Uncertainty of 2 ml pipette volume

A 2.0 ml pipette was also used to pipette 6.0 ml of stock solution for second standard. The following components of uncertainty are taken into account:

1. Tolerance or calibration of the volume as specified by the manufacturer
2. Uncertainty due to the repeatability of pipetting
3. Uncertainty of the volume due to temperature difference from 20 °C

The calculation assumes that the pipette has been thoroughly cleaned, and that no droplets are formed on the inner surface of the pipette after draining the liquid.

Model Equation:

3.6.1 Volume of the 2 ml pipette

Table 7: List of Uncertainty Quantities for the 2 ml pipette:

Quantity	Unit	Definition
V_{1_2}	ml	Volume of the 2 ml pipette
$V_{1\_2\_cal}$	ml	Value of the volume together with calibration uncertainty contribution
$V_{1\_2\_repeat}$	ml	Uncertainty contribution of the volume due to repeatability
$V_{1\_2\_temp}$	ml	Uncertainty contribution of the volume due to temperature difference from 20 °C
γ	1/ °C	Thermal expansion coefficient of water
Δt	°C	Difference of liquid temperature from 20 °C

The volume is expressed as sum of three components. The V_{tol} carries the value together with calibration uncertainty.
The other two components carry only uncertainties, their values are set to zero.

$$V = V_{tol} + V_{repeat} + V_{temp}$$

Uncertainty of the pipette volume due to temperature difference from 20 °C

$$V_{1\_2\_temp} = V * \gamma * \Delta t$$

3.6.2 $V_{1\_2\_cal}$: Type B rectangular distribution
Value: 2.00 ml
Limits: ± 0.010 ml

U $_{cal}$: $U_{cal} = \frac{0{,}010}{\sqrt{3}} = 0{,}0057735$

3.6.3 $V_{1\_2\_repeat}$: Type A summarised
Mean: 0 ml
Standard Uncertainty: 0.008 ml

It may be estimated here that the repeatability standard uncertainty of pipetting is 0.4% of the pipette volume. This value may be assumed as a safe estimate.

U_{repeat}: $U_{repeat} = \frac{0,008}{\sqrt{3}} = 0,0046188$

3.6.4 $V_{1\_2\_temp}$ Interim result

γ: Constant
Value: 0,00021 1/°C

Δt: Type B rectangular distribution
Value: 0 °C
Limits: 3 °C

It is reasonable to assume that the temperature in the lab does not differ from 20 °C by more than 3 degrees.

$V_{1\_2\_temp} = V * \gamma * \Delta t$

$= 2 \times 0.00021 \times 3$ °C

$= 0.00126$ ml

$U_{Temp} = \frac{0.00126}{\sqrt{3}} = 0,0007274$

3.6.5 Combined Uncertainty of 2 ml volumetric pipette using worst case:

$$U_{Volumetric} = \sqrt{(U_{cal})^2 + (U_{Repeat})^2 + (U_{Temp})^2}$$

$$= \sqrt{(0{,}0057735)^2 + (0.0046188)^2 + (0.0007274)^2}$$

$$= 0{,}00743$$

3.7 Combined Uncertainty of the Standard solution

The Combined uncertainty of the Standard solution can, therefore, be expressed as a quotient using uncertainty parameters associated with the preparation of the standard solutions used in the assay, that is: weighing a Certified Reference Material (CRM) on a balance into a volumetric flask as stock solution and subsequently diluting it to prepare the remaining standard solutions.

$$U_{Std} = \text{mass weighed} \sqrt{\left(\frac{U_{Purity}}{Purity}\right)^2 + \left(\frac{U_{Volume}}{Volume}\right)^2 + \left(\frac{U_{Weigh}}{Mass}\right)^2 + \left(\frac{U_{Pipette}}{Pipette}\right)^2}$$

$$= 125{,}8\sqrt{\left(\frac{0{,}000577}{1{,}00}\right)^2 + \left(\frac{0{,}02138}{25}\right)^2 + \left(\frac{0{,}0291}{10}\right)^2 + \left(\frac{0{,}18607}{125}\right)^2 + \left(\frac{0{,}0127}{4}\right)^2 + \left(\frac{0{,}00743}{2}\right)^2}$$

$$= 0{,}4686$$

3.8 Uncertainty of the sample solution

The measurand is expressed in the units of g/l. The solution was injected directly without any preparation or dilution to it. Therefore the only uncertainty component to evaluate would be that from the instrument.

3.9 Uncertainty of Instrumental Operations

The values for injector and flow rate variance were obtained from Performance Qualification done by an Accredited Waters Compliance Specialist in July 2006. Assuming a rectangular distribution:

3.9.1 Injection of 20 µl = 20 µl ± 1.0 µl

1.0 ± 0.05

$$U_{Inj} = \frac{0.05}{\sqrt{3}} = 0.02887$$

3.9.2 Flow rate of 1.0 ml/min = 1.0 ml/min ± 0.012 ml/min

= 1.0 ± 0.012

$$U_{Flo} = \frac{0.012}{\sqrt{3}}$$

= 0.006928 ml/min

3.9.3 Values for repeatability of sodium lactate were taken from the validation data of the specific method.

Table 8: Repeatability values of sodium lactate from validation data

3,079 g/l
3,027 g/l
3,062 g/l
3,043 g/l
3,063 g/l
3,077 g/l

Average = 3.0585 g/l sodium lactate

s = 0,02014 (6 determinations)

$$ESDM = \frac{s}{\sqrt{n}} = 0,00822$$

3.9.4 Combined Uncertainty of Instrumental Operations using worst case

The combined uncertainty of the Instrument can therefore be expressed as a quotient using uncertainty parameters associated with the operation of HPLC/UV used in the assay, that is: injection of equal volumes of a standard and sample at a set flow rate and detection at a fixed wavelength.

$$U_{Inst} = \sqrt{(U_{Inj})^2 + (U_{Flo})^2 + (U_{Repeat})^2}$$

$$U_{Inst} = \sqrt{(0,02887)^2 + (0,006928)^2 + (0,00822)^2}$$

$$= 0,0308065$$

3.10 Overall Expanded Uncertainty

The overall expanded uncertainty is calculated by incorporating the combined standard uncertainties associated with the standard preparation as well as the combined HPLC instrumental standard uncertainties calculated above.
The final stage is to multiply the combined standard uncertainty by the chosen coverage factor in order to obtain an expanded uncertainty. [5]
The expanded uncertainty is required to provide an interval which may be expected to encompass a large fraction of the distribution of values which could reasonably be attributed to the measurand.
For this study $k = 2$ and a 95% confidence level is assumed.

$$U_{Expanded} = 2 \times \sqrt{(U_{Stnd})^2 + (U_{Inst})^2}$$

$$= 2 \times \sqrt{\left(\frac{0,4686}{125}\right)^2 + \left(0,0308065\right)^2}$$

$$= \mathbf{0,0621 g/l}$$

Result: Quantity: $C_{\text{Sodium Lactate}}$

Value from analysis: 2,93 g/l

Expanded Uncertainty: ± 0,0621 g/l

Coverage factor: 2,00

Results of Sodium lactate can now be reported with an uncertainty value accompanied by an uncertainty statement.

3.11 Uncertainty statement:

The 'true" content of sodium lactate in the solution is in the range 2,87 – 2,99 g/l with 95% probability (in the case of normal distribution).

This range is well within the specification limit of 2,85 – 3,15 g/l.

4. Discussion

4.1 The contribution of uncertainty components: An overview

The uncertainty originating from sample preparation is considered negligible and gives no contribution to the combined uncertainty. The sample is a clear homogenous solution and sodium lactate is completely soluble in water. No sample preparation was necessary and the sample was injected as is.

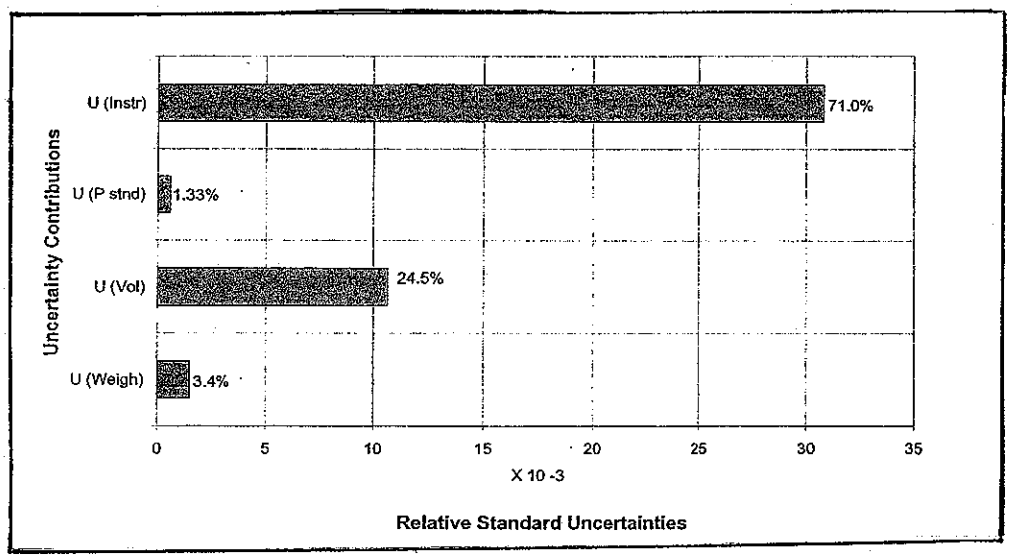

Fig 4: Uncertainty budget indicating uncertainty contributions

The uncertainty components from weighing measurements were small. In fact the 3,4 % uncertainty contribution of weighing is even somewhat larger than usual. Mass uncertainty comes from the uncertainty of weighing. With the digital balance a mass of substance is found from the difference of results of two weighing operations: first volumetric flask is weighed, then the balance is tared and after that the substance is transferred into the flask and the weight is registered.

We determined the weighing repeatability (instead of using the data from the calibration certificate), in order to mimic the weighing procedure as accurately as possible. The above procedure was carried out 20 times and the mean and standards deviation was obtained. Thereafter a standard uncertainty of repeatability was obtained = 0,02133 mg.

The drift and readability components were estimated as Type B distributions.

The uncertainty contribution due to volume is somewhat larger than usual. This is because two pipettes of 4 ml and 2 ml each were used to dispense a volume of 6 ml instead of a single 6 ml pipette. This therefore introduced more error and increased the uncertainty contribution due to volume.

The uncertainty contribution from instrumental measurements was much higher (71%) than the contributions from the measurements of the other uncertainty sources identified. As a result, in order to improve the method, all the efforts should be directed towards decreasing the uncertainty of instrumental measurement. For example, revalidating the instrument, checking flow rates, checking injection volumes and ensuring system suitability criteria are met prior to performing an analysis.
The standard uncertainties of the peak areas were found using validation data. An average of 3,0585 g/l was obtained. This could be done as values were obtained using the same method but on different days.
The repeatability component takes into account the repeatability of injection, random variations in mobile phase flow rate and composition, repeatability of the absorbance detector, repeatability of detector wavelength.

4.2 Cross contamination:

This uncertainty component is quite difficult to take into account, while its value depends on previous injection. In this work we chose to show the negligibility of this effect and leave it out. This is achieved by using triple injection series for repeatability studies. If the same solution is injected three times in a row, then in the case of considerable cross contamination the area of first injection should be noticeably different from the following ones. Based on the data obtained, one can see that this is not the case (there is no difference between the first and the following injections in a group), thus it can be said that this component is not likely to contribute a considerable contribution to uncertainty. This assumption is also supported by the following two considerations:

a) Cross contamination is a problem mainly when an on-line filtration of solutions is used. But in this work all the solutions were filtered into autosampler vials, so additional on-line filtration was not necessary.
b) Cross contamination has larger uncertainty contribution in trace analysis. In this study the concentrations analysed were relatively high.

4.3 Dissolved oxygen content in the mobile phase:

This is a subtle and often neglected cause of problems in chromatographic analysis. It can be an important cause of degradation of quality of results. Dissolved oxygen absorbs UV radiation at 210 nm and lower, and if detection is carried out in that region, then dissolved oxygen can cause fluctuations and drift of the baseline. In addition – dissolved oxygen may react with the analyte. These reactions cause drift and variability of results. This is especially important in trace analysis. In this work an on-line degasser was employed, which brings the dissolved oxygen level to a minimum. No anomalous fluctuations or drift was detected as seen in validation data. Based on these considerations there were no grounds for the introduction of a separate uncertainty component, especially keeping in mind that small fluctuations and drift are taken into account already with the repeatability and drift components.

The nonlinearity uncertainty is taken into account only with the peak area of the sample. This is an uncertainty component caused by the use of linear calibration in the case of slightly non-linear calibration curve. R for this method was 0,9999999. Non-linearity of the calibration graph is a systematic effect that, in principle, should be corrected. At the same time the only logical way to do this would be to utilise non-linear regression. This would increase the complexity level of this method making it harder to use.

In addition it is not clear how large the contribution of nonlinearity to the whole uncertainty is, and is the decrease in uncertainty noticeable after the correction.

The uncertainty estimation in this study has provided great insight into how a measured result may be affected by parameters, which previously were taken for granted. Measurement of volumes, masses, environmental temperature differences on volumes, calibrations and instrumental conditions all have an effect on measured and ultimately the accuracy of reporting a final result.

Previously results were reported without any level of confidence. Now reporting a result with an uncertainty value reassures that the true value lies within the specified range with a 95 % confidence level.

5.0 Conclusion

An ISO GUM measurement uncertainty estimation procedure was developed for a liquid-chromatographic drug quality control method - Assay of Sodium Lactate in sodium lactate solutions.

In quantification of uncertainty components, several practical approaches for including difficult –to- estimate uncertainty sources (such as uncertainty due to peak integration, uncertainty due to non-linearity of the calibration curve, etc.) have been presented.

Detailed analysis of contributions of the various uncertainty sources was carried out. The results were calculated based on the definition of the measurand and it was demonstrated that unequivocal definition of the measurand is essential in order to get rigorous uncertainty estimate.

The main conclusions from the analysis of the uncertainty budget are the following:

1) The uncertainty originating from sample preparation is negligible to the combined uncertainty.
2) The uncertainty components from weighing and volume measuring were small.
3) The uncertainty contribution due to non-linearity is insignificant, as most of the uncertainty sources related to calibration are of systematic nature with respect to the different points on the calibration graph and thus increase of the number of points does not lead to a noticeable decrease in uncertainty.

Results of Sodium Lactate can now be reported with an uncertainty value accompanied by an uncertainty statement. The value of 0,0621 mg/ml can now be incorporated when reporting a sodium lactate result on a finished product containing sodium lactate in solution. For example, in this study, a value of 2,93 mg/ml was found. The final result may be reported as:

2,93 ± 0,0621 mg/ml, which means:

The 'true" content of sodium lactate in the solution is in the range 2,87 – 2,99 g/l with 95% probability (in the case of normal distribution).

6.0 REFERENCES

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