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Method of Estimating Uncertainty of Measurement Values in Case of Measurement Methods Involving Non-Linear Calibration

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Summary

Expressing the reliability of measurement results by using uncertainty is becoming the international rule. We have reported on an uncertainty estimation method for the routine test data and assigned values of calibrator by differentiating the calibration procedures for the measurement methods into those that are performed each time and those that are performed at certain intervals. This paper discusses the measurement methods whose calibration curve are not linear and presents the methods for estimating the uncertainty of these measurements by means of examples, including specific procedures. While measurements involving non-linear calibration curve, such as immunochemical analysis methods, have recently been used on an extensive scale the fact remains that the methods of expressing the reliability of the measurement data have not been sufficiently examined. We therefore take this opportunity to present here a method for estimating uncertainty based on repeat measurement values, using as samples the reference materials employed for the preparation of the calibration curve.

1. Introduction

The method of expressing the reliability of measurement results is outlined in the International Guide to the expression of uncertainty in measurement (GUM)[1)] and is rapidly expanding mainly in the area of quantitative measurement, including analytical chemistry and applied physics.

Similarly in the field of clinical testing, a number of methods such as the Method of setting the uncertainty of calibrator assigned values[2)] and the estimation method for the uncertainty of routine examination results[3)] have been reported and come to be discussed in specific detail in conjunction with the introduction of the Medical Laboratory Accreditation System based on the ISO standard 15189 [4)] on medical laboratory quality and competence.

In recent years, however, immunochemical analysis methods have been widely used for clinical examination. One of the unique features of these methods is the fact that the calibration curves are not linear. There has been almost no opportunity so far to assess in specific detail the evaluation methods for the reliability of the measurement data obtained from such quantitative analyses. This report thus focuses on the estimation of the uncertainty of measurement data obtained from measuring methods that involve non-linear calibration.

2. Evaluated Measurement Methods

The analysis methods presented here are used in the event that the uncertainty of measurement results is determined when the value of the measurand is quantitatively determined by absorptiometry. When the relationship between the measurand value and the analysis output value (absorbance) is non-linear, a plurality (3 or more) of reference materials with different values is used to determine the formula for the relation between these known values and the absorbance (working curve). The measurement values for estimating the values for the unknown samples are obtained by reverse calculation with the absorbance values being introduced into the relational formula.

Provided that calibration is performed in the correct manner, the relation between the measurand value (x) and the measured value (y) will be a linear function (ideally, a function of type $y = x$). This rests on the prerequisite condition that the relation between the measurand value and the measured absorbance is mathematically treated in an appropriate manner and that the reliability of the measurement values obtained with the use of these working curves is guaranteed. For the measurement methods used in the medical laboratory it is reasonable to suppose that their validity is assured. In this sense, it is legitimate to treat the relation between the values of the reference materials and the measured values as a linear function independently of the shape of the working curves, provided that calibration is performed in the appropriate manner.

3. Classification and estimation of components of uncertainty

Although there is a large diversity of components of uncertainty in chemical analysis such as sample weight and volumetry, measuring procedures, reference materials and interfering substances[2-5] these can be divided into three main categories in case of absorptiometry. The magnitude of their uncertainties can be estimated in accordance with the GUM procedures.

(1) Uncertainty of Reference materials (Standard uncertainty = us, or its relative value)
Uncertainty of certified values includes stability and uniformity. Normally, this is specified on the certificate based on type B evaluation.

(2) Uncertainty associated with Calibration (Standard uncertainty = u_B)
In immunochemical analysis, the practice is in some cases that calibration is performed using reference materials in circumstances such as the change of a reagent lot, and that after this

measurement is continued for a certain time without calibration when conditions are considered as almost constant. In these instances, it is necessary to estimate uncertainty by differentiating the uncertainty due to calibration from the uncertainty associated with the measuring operations.

Uncertainty due to calibration is estimated by measuring, as samples, the reference materials used for establishing the calibration curve and by applying analytical methods that utilize the SN ratio for the relation between the assigned values and the measured values[6-7].

(3) Uncertainty associated with the measuring procedures (Standard uncertainty = u_{CAL}, or the relative value thereof)
This uncertainty includes the factors due to within-day and between-day variations for the measurement units, the variations in the samples and sample preparation, reagent preparation and the equipment. When calibration is performed with each measurement the uncertainty due to calibration must also be included. These uncertainties are generally estimated on the reproducibility experiments based on type A evaluations.

Uncertainty associated with the measuring procedures involves estimation of the uncertainty of between-day variations (u_A) and within-day variations a(u_E) by applying analysis of variance to the repeat measurement values from the samples. By synthesizing these factors, it is possible to determine the standard uncertainty associated with the measuring operations ($u_M = (u_A^2 + u_E^2)^{1/2}$).

(4) Combined uncertainty of measurement values (Standard uncertainty = u_C, or its relative value)
By using each estimation value for the uncertainty of the reference materials obtained, the uncertainty due to calibration and the uncertainty associated with the measuring operations, it is possible to obtain the combined standard uncertainty in case of single measurement for routine testing as follows:

$$u_C = (u_S^2 + u_{CAL}^2 + u_M^2)^{1/2}$$

Used as the reference materials are substances having a similar matrix to the patient samples such as reference sera and no sample matrix effect and no effect due to interfering substances shall be detectable.

4. Estimation of Uncertainty due to Calibration

A measurement method is calibrated using a reference material of level m (≥ 3) whose measurand value is x_i (where $i = 1,2,...,m$). Immediately after calibration, the measured value shall be y_{ij} (where $i = 1,...,m$; $j = 1, ...,n$)when the measurements of the reference materials are repeated n times. This operation is also called “strike-back.” Its significance lies in the fact that it verifies whether calibration has been performed appropriately. We will here deal with the case in which a rectilinear relation $y_{ij} = \alpha + \beta x_i + \varepsilon_{ij}$ exists between x and y. Thus, α contains the slope of the zero point, β the

slope of the verified calibration line (called the sensitivity coefficient) and ε_{ij} is the error including the discrepancy from linearity, it is assumed that the ε_{ij} are normally distributed with variance σ^2 .

The error variance can be calculated from the measured values for the reference materials using the SN ratio as follows when calibration has been carried appropriately[6-7]. The total variation S_T is split into three parts: the variation S_B due to the measured quantity, the magnitude S_m of the generalized mean effect and the error variation S_e (i.e., $S_T = S_B + S_m + S_e$). These variations, effective divisors, and error variance V_e are calculated as follows.

$$S_T = \Sigma\Sigma y_{ij}^2, \quad S_m = (\Sigma\Sigma y_{ij})^2/(mn), \quad r = n\Sigma(x_i - XB)^2,$$
where $(XB = (1/m)\Sigma x_i, \quad S_B = \{\Sigma(x_i - XB)\, y_i\}^2/r,$
where $y_i = \Sigma y_{ij}, \quad S_e = S_T - S_m - S_B, \quad V_e = S_e/(mn - 2).$

Based on this, the SN ratio (η) can be calculated using the following equation:

$$\eta = \beta^2/\sigma^2 = (1/r)\cdot(S_B - V_e)/V_e$$

The SN ratio is determined by dividing the square of the magnitude of the calibration line's sensitivity by the error variance. The reciprocal of the SN ratio becomes the estimated value of the error variance of the measurement values when calibration has been performed correctly. Using the SN ratio, it is possible to obtain the uncertainty due to calibration as follows:

$$u_{CAL} = 1/\sqrt{(\eta)}$$

5. Estimation of Uncertainty Associated with Measurement

The uncertainty associated with measurement procedures can be determined by taking reference materials or control materials as the samples and by applying the Analysis of V**a**riance Method[8] to the repeat measurement data with the between-day and within-day as factors. Analysis of variance was then applied to each level of the reference material to obtain the uncertainty.

In other words, by carrying out n-times repeated measurements of the sample material during the testing time of p days (times) it is possible to obtain p x n measurement values (z_{ij}, where $i = 1,...,p$; $j = 1,...,n$). The preferable number of days of measurement (number of times) should be 15 or more, and the minimum repeat number per measurement day should be 2 [9]. After checking that there is no outlier among the measurement values, analysis of variance is applied to determine the total variation S_T, the between-day variation S_A and the within-day variation S_E.

$$S_T = \Sigma\Sigma(z_{ij} - ZB)^2, \quad S_A = n\Sigma(ZB_i - ZB)^2, \quad S_E = \Sigma\Sigma(z_{ij} - ZB_i)^2 = S_T - S_A$$

where ZB is the total mean measurement value for the tested sample material and ZB_i the mean value per day. The unbiased estimate of variance for between-day and within-day variations V_A and V_E can be determined as follows:

$$V_A = {}_A / (p - 1), \quad V_E = S_E / \{p(n - 1)\}$$

From the above, the estimation values for the between-day uncertainty due to measurement u_A and the within-day uncertainty u_E can be calculated and the combined standard uncertainty associated with measuring procedures u_M can be determined.

$$u_A = \{(V_A - V_B) / n\}^{1/2}, \quad u_E = V_E^{1/2}, \quad u_M = (u_A^2 + u_E^2)^{1/2}$$

Moreover, for the statistical analysis required for uncertainty estimation the special software provided by the Japanese Committee for Clinical Laboratory Standards (JCCLS) can be used. The latter is available from the homepage (URL: http://www.jab.or.jp) of the Japan Accreditation Board for Conformity Assessment.

6. Example of Uncertainty Analysis for Measurement Values Based on Routine Test Methods with Non-linear Calibration

The following is an example of the manner in which the uncertainty of serum CRP measurements based on the latex immune turbidity comparison method with non-linear calibration is estimated. The analysis is carried out using an automatic analyzer. Two types of reagent were added at 120μl each to 2.4μl of the sample is made and absorbance is measured after a reaction time of 5 minutes. For calibration, and 6 levels of reference material (0.0, 3.0, 6.0, 30.0, 180.0, and 420.0mg/l) are used and the calibration curves are processed by Spline function approximation. The values for the reference materials are traceable to IFCC International Plasma Protein Reference Standard IRMM-CRM470, and the relative value for the standard uncertainty of the assigned value is assumed to be constant at 5.0%.

As seen in this example, the immunochemical analysis method has a wide measurement range and the magnitude of the variability also differs according to the value. In this case, we estimate the reliability within the range limited to a value of 30.0mg/l or less.

First, in order to estimate the uncertainty due to calibration repeat measurements are performed at random four times for the 4 levels of reference materials (0.0, 3.0, 6.0, and 30.0mg/l) used for calibration. The measurement values thus obtained are shown in Table 1.

From the data in Table 1, it is possible to calculate the total variation S_T, the variation due to the measured quantity S_B, the magnitude of the generalized mean effect S_m, the error variation S_e, as

well as the effective divisor r and the error variance V_e, and from these values it is then possible to obtain the SN ratio (η) and the standard uncertainty due to calibration (u_{CAL}) as follows.

$$\eta = (1/r)\cdot(S_B - V_e)/V_e = 30.04, \quad u_{CAL} = 1/\sqrt{(\eta)} = 0.18$$

The SN ratio and uncertainty determined from these four types of measurement values by selecting only three levels of reference material (0.0, 3.0, and 6.0mg/l) assume the following values:

$$\eta = (1/r)\cdot(S_B - V_e)/V_e = 115.37, \quad u_{CAL} = 1/\sqrt{(\eta)} = 0.09$$

It can be seen also from a comparison of these two values that the reliability of the CRP measurement varies in accordance with the measurement concentration. One method to address this variation is to either make the evaluation separately for each concentration region for which it can be assumed that the magnitude of variation does not change so significantly or to make an evaluation after checking that the magnitude of variation will be constant by a procedure such as the logarithmic transformation applied to the measurement values. Here we will use the calculation results based on the four types of reference materials for later processing.

Next we repeated double measurements of the three levels of reference material (3.0, 6.0 and 30.0mg/l) each day for 20 days. This yielded the measurement values shown in Table 2. Analysis of variance was then applied to each level of the reference material to obtain the uncertainty for between-day variations (u_A), the uncertainty for within-day variations (u_E) and the combined standard uncertainty associated with the measuring operations (u_M). These values are presented in the lower part of Table 2. It is possible to use also the universal table calculation software Microsoft Excel for the one-way layout variance analysis used in this case.

The relative value of 5.0% for the uncertainty of the assigned values for the reference material was combined with the above uncertainty due to calibration and the uncertainty associated with the measuring operations and the combined standard uncertainty and extended uncertainty were obtained as follows for each CRP measurement concentration region (with the coverage factor being $k = 2$).

3.0mg/l: $\{(3.0 \times 0.05)^2 + 0.18^2 + 0.07^2\}^{1/2} = 0.24$, Extended uncertainty: ± 0.5mg/l
6.0mg/l: $\{(6.0 \times 0.05)^2 + 0.18^2 + 0.16^2\}^{1/2} = 0.38$, Extended uncertainty: ± 0.8mg/l
30.0mg/l: $\{(30.0 \times 0.05)^2 + 0.18^2 + 0.42^2\}^{1/2} = 1.57$, Extended uncertainty: ± 3.1mg/l

The uncertainties of the measurement values for the patient samples in the respective CRP concentration regions can thus be stated as shown above.

7. Conclusion

With regard to measurement methods involving a non-linear calibration curve, a method has been proposed for estimating uncertainty from repeat measurement values using as samples the reference materials employed for preparing the calibration curve. If the magnitude of the uncertainty differs exceedingly according to the measurement concentration in this case, it is necessary to determine an estimation value corresponding to the measurement concentration by adequately allowing for the particular conditions. Although the basic procedures used to estimate uncertainty are quite simple and clear as shown in the GUM, the key factor in estimating the uncertainty of measurement lies in how to appropriately identify and evaluate the various components of uncertainty that are involved in the measurement process, including the calibration procedures.

References

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Table 1　Repeatability of reference materials after calibration

Repeat	Reference materials （mg/l）			
	0.0	3.0	6.0	30.0
1	0.0	2.9	5.9	29.8
2	0.0	3.0	5.8	30.3
3	-0.1	3.0	6.1	30.6
4	0.0	2.9	6.1	30.5

Table 2 Reproducibility of reference materials of duplicate measurements for 20 days

Day	Measurement values of reference materials					
	3.0 mg/l		6.0 mg/l		30.0 mg/l	
1	3.0	3.0	5.8	5.8	30.6	30.5
2	3.1	3.0	6.2	6.0	30.1	30.1
3	3.0	3.0	6.0	6.0	30.6	30.1
4	3.1	3.0	6.3	6.0	30.6	30.6
5	3.0	3.1	6.0	6.0	31.1	30.3
6	3.1	3.0	6.2	6.3	31.3	31.7
7	3.1	3.1	6.3	6.2	31.1	30.9
8	3.1	3.0	6.4	6.3	31.0	30.8
9	2.9	3.0	6.0	6.0	30.5	29.9
10	3.0	3.0	6.0	6.1	30.8	31.0
11	3.1	3.1	6.1	5.8	30.2	30.3
12	3.0	3.0	5.7	5.9	30.3	30.8
13	3.2	3.1	6.2	6.3	31.0	30.9
14	3.0	3.0	6.2	6.2	31.0	31.0
15	3.0	2.9	6.0	6.2	30.3	30.3
16	3.0	3.0	6.2	6.0	30.5	30.2
17	3.0	2.9	6.1	6.3	30.8	29.8
18	2.9	3.0	6.2	6.1	31.2	30.7
19	3.0	3.1	6.1	6.1	30.9	30.4
20	3.0	3.0	6.1	6.2	30.6	30.0
Mean	3.02		6.10		30.62	
Between-day (u_A)	0.04		0.12		0.29	
Within-day (u_E)	0.05		0.11		0.31	
Total var. (u_M)	0.07		0.16		0.42	
CV (%)	2.2		2.7		1.4	