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Estimation of uncertainty in kinetic-colorimetric assay of bacterial endotoxins

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Abstract

The relative standard deviation (R.S.D.) of measurements is estimated in the kinetic-colorimetric assay of bacterial endotoxins without recourse to the usual repeated experiments. The measurements are the slopes of kinetic curves and two major factors are considered to cause the uncertainty of the measurements: (1) the pipetting of the sample and color development reagent; and (2) noise in the detection unit. The measurement R.S.D. is formulated as a function of endotoxin concentration. Two parameters (S.D. of the pipetted volumes and S.D. of the detector noise) are also required in the uncertainty equation, but no arbitrary coefficients are included. Since the S.D. values for pipettes and detector noise can be determined independently of the endotoxin assays, the measurement R.S.D. can be estimated by the above equation without repeating the assays. However, the calibration curve is necessary. The theoretical estimation is shown to be in good agreement with the experimental R.S.D. ($n = 12$) over a wide concentration range.

Keywords: Endotoxin; LAL test; Kinetic-chromogenic assay; Method validation; Precision

1. Introduction

The endotoxins of Gram-negative bacterial origin are the most relevant substances inducing hyperthermia in humans. Since the bacterial endotoxins can contaminate pharmaceuticals and medical devices, the purity and safety should be assured [1–5]. Endotoxin tests of wide international acceptance utilize the blood corpuscle extracts of horseshoe crabs (limulus amebocyte lysate; LAL) [6–8].

The LAL has various endotoxin-sensitive clotting factors and a firm gel is formed after the factors are activated at the cascade of the reaction of the endotoxins with the LAL. The gel clotting potency is in proportion to the endotoxin concentration, enabling the detection as well as quantification of the endotoxins (gel-clot technique and turbidimetric technique). If a synthesized substrate, which can release a chromophore at the end of the cascade reaction, is contained in the LAL reagent, the absorbance of the chromophore

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can be related to the endotoxin concentration (colorimetric technique).

In general, the uncertainty of measurements has been recognized as the main unifying principle in data quality [9]. It is defined as an estimate characterizing the range of values within which the true value of analyte concentration lies [9] and is expressed as S.D. and R.S.D.

This paper focuses on the uncertainty of the kinetic-colorimetric assay which measures the rate of color development throughout an incubation period. Unfortunately, the number of wells of a microplate as well as the price of LAL reagents restricts the number of repeated measurements in the endotoxin assay and in turn, makes it difficult to estimate the exact value of S.D. from the repeated measurements. Statistics tells that the reliability of the estimated S.D. cannot be high until measurements are repeated a sufficient number of times ($=n$). For example, the 95% confidence intervals of estimated S.D. are $\approx \pm 60\%$ of the true value for $n=6$, but $\approx \pm 20\%$ for $n=40$. The latter reliability is acceptable, but the number ($n=40$) is not feasible for practical purposes.

The aim of this paper is to put forward a probabilistic method for estimating the uncertainty without repeated measurements, but with satisfactory reliability in the kinetic-colorimetric assay. To achieve the purpose, we choose a part of the assay procedures that affect the measurement uncertainty. The assay is very simple:

Step 1: the addition of the LAL reagent and sample into the wells of a microplate;
Step 2: the least squares fit of a straight line to the reaction curve in the incubation period.

Here, the measurements are the slopes of the observed reaction curves. Therefore, the influential factor of step 1 is the error of the pipetting and that of step 2 is the reproducibility of the least squares fitting of a straight line to the noisy reaction curve.

This paper describes the measurement R.S.D. for the endotoxin assays as a function of endotoxin concentration, S.D. of pipetting and S.D. of detector noise. The validity of this theoretical estimation is experimentally verified.

As far as the instrumental analyses, such as liquid chromatograph and atomic absorption spectrometry, are concerned, the study on the theoretical estimation of measurement uncertainty has been carried out for many decades [10–22]. To the best of the authors' knowledge, however, no relevant publications on the endotoxin assays could be found.

2. Materials and method

The LAL kinetic-chromogenic assay was performed according to the endotoxin test in Japanese Pharmacopoeia XIV edition [6]. The endotoxin-specific LAL reagent (Endospecy) was purchased from Seikagaku Kogyo, Tokyo, Japan. An endotoxin solution (50 μl) was mixed with the same volume of LAL solution containing chromogenic substrate in a 96 well flat microplate, shaken for 1 min and kept at 37 °C for 30 min. During the incubation period, the absorbance was measured with a microplate reader (Wellreader SK601, Seikagaku Kogyo) at 405 and 492 nm simultaneously. The measurements (slopes) were expressed as the absorbance (405 nm) per minute (mAbs/min) with 492 nm as a reference. The Japanese Pharmacopoeia Endotoxin 100 Standard was used as a standard in the assay. The sampling intervals were 15 s.

Six blank data are averaged at each data point from 40 to 120 (10–30 min) and a straight line is least-squares fitted to the averaged data. The fitted straight line is considered a blank line and is subtracted from each reaction curve. All the analyses in this paper are based on the blank–subtracted curves. For example, the noisy reaction curves of Figs. 1 and 2 are the blank–subtracted curves and Y-axis of the calibration line is the slope of a straight line which is least-squares fitted to the blank–subtracted curves.

In Figs. 3–5, the solutions of p-nitroaniline (PNA), that is the chromogen of the LAL reagent, were used instead of the endotoxin assay itself. This reflects our opinion that the uncertainty of the endotoxins' test can be estimated from separate experiments.

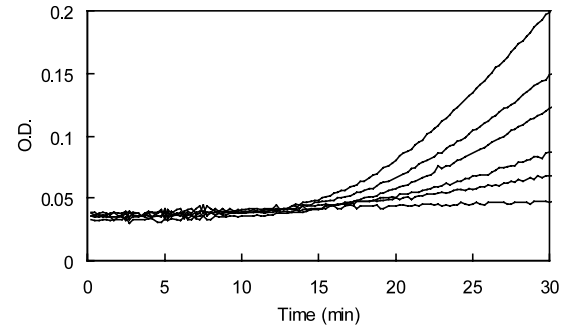

Fig. 1. Time courses of kinetic endotoxin assay. Concentrations (from bottom to top lines): blank, 0.0078, 0.0156, 0.0313, 0.0625, 0.125 EU.

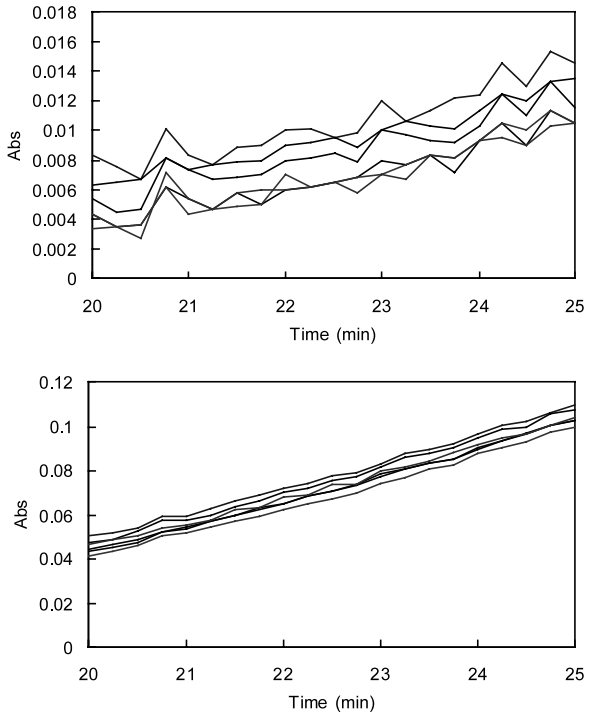

Fig. 2. Time courses used for calibration and analysis. Concentrations: 0.0078 (top); 0.125 (bottom). This region includes 21 data points (sampling intervals are 15 s). The blank reaction curve, which is the least squares fit of a straight line to the averaged blank lines, is subtracted from the reaction curves of this figure.

Fifty micro-liters of distilled water (or BSA solution) were taken by a Gilson pipette and transferred into a small beaker placed on an electronic balance. This procedure was repeated 40 times and the statistics of the results are listed in Table 1. Some 20 mg/ml BSA solution (50 μl) corresponding to the protein concentration of the LAL reagent was used to examine the effect of the viscosity of the solution on the pipetting uncertainty.

3. Theory

3.1. Uncertainty of mixture

We derive an equation to describe the uncertainty of absorbance measurements, A, in the plate reader when two solutions are mixed in a well of a microplate. It is assumed that one solution has an absorbing material and the other has no absorbance and that the detector noise is neglected in Eqs. (1) and (2). In practice, a well in the plate has absorbance of its own. Then, we consider two affecting factors on absorbance measurement, A, in the well reader: (1) absorbance, W, inherent in a well; (2) volume, V, of absorbing solution A taken by a pipette or dispenser. From this assumption, it follows (Appendix A):

$$(S_A)^2 = (S_W)^2 + \left(\frac{S_V}{V}\right)^2 A^2 \quad (1)$$

$$(R_A)^2 = \left(\frac{S_W}{A}\right)^2 + (R_V)^2 \quad (2)$$

where S_X denotes the S.D. of quantity X and R_X, the R.S.D. of X. The quantities on the right sides of Eqs. (1) and (2) can be estimated by experiments and we will examine the correctness of the equations later (see Fig. 4).

3.2. Uncertainty of endotoxin assay

The measurements in endotoxin assays are the slopes of the reaction curves of the PNA production and make the Y-axis of the calibration line. The slope measurements are obtained by the least squares fitting of a straight line to the noisy reaction curves. The major error sources of the slope measurements are assumed to be:

i) the change in the apparent reaction rates;

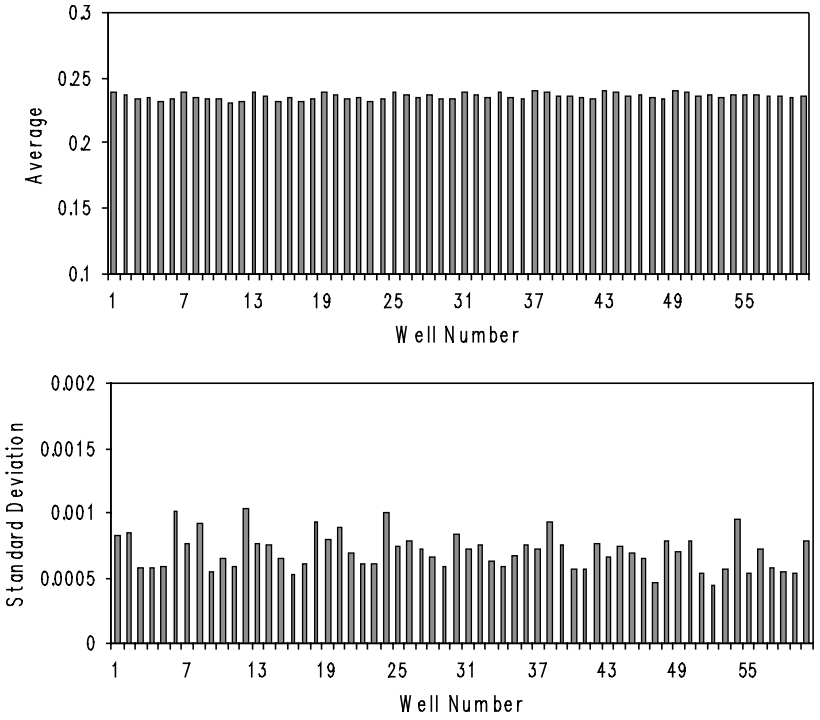

Fig. 3. Statistical performance of microplate. A hundred microliters of PNA solution are pipetted into each well of a microplate. The edge wells are not used and the examined wells total to 60 ($=6\times10$). The pipetting is not repeated, but the average and S.D. are obtained from 121 times light detection a well.

ii) the least squares fitting of a straight line to the observed time variation.

In our analytical system, the noise comes from the detector of the plate reader.

By taking into account the rule of error propagation [23–25], we can describe the total error originating from sources (i) and (ii) as:

$$(S_T)^2 = (S_r)^2 + (S_l)^2 \tag{3}$$

where S_T denotes the S.D. of the slope measurements; S_r, the S.D. of the slopes depending on the apparent reaction rates (source i); and S_l, the S.D. of the slopes estimated by the least squares fitting (source ii). The dimension of both sides of the above equation should be the same (Abs/time). By dividing both sides of Eq. (3) by the mean of the slope measurements, we obtain the R.S.D. of the slope measurements as:

$$(R_T)^2 = (R_r)^2 + (R_l)^2 \tag{4}$$

where R_T, R_r and R_l are the R.S.D.s corresponding to S_T, S_r and S_l, respectively.

We can derive the objective equation (see Appendix B):

$$(R_T)^2 = 5(R_V)^2 + \frac{\tilde{e}^2}{\left(\sum_{i=1}^{N}(X_i - \bar{X})^2\right)} \times \frac{1}{\bar{a}^2} \tag{5}$$

where R_V denotes the R.S.D. of volumes taken of endotoxin solution ($=$ LAL solution), $\tilde{e}$, the S.D. of the detector noise, X_i, the time or data point at which the ith slope measurement is obtained for the least squares fitting, the mean of X_i over i and $\bar{a}$, the mean of slope measurements which in practice can be replaced by the calibration line.

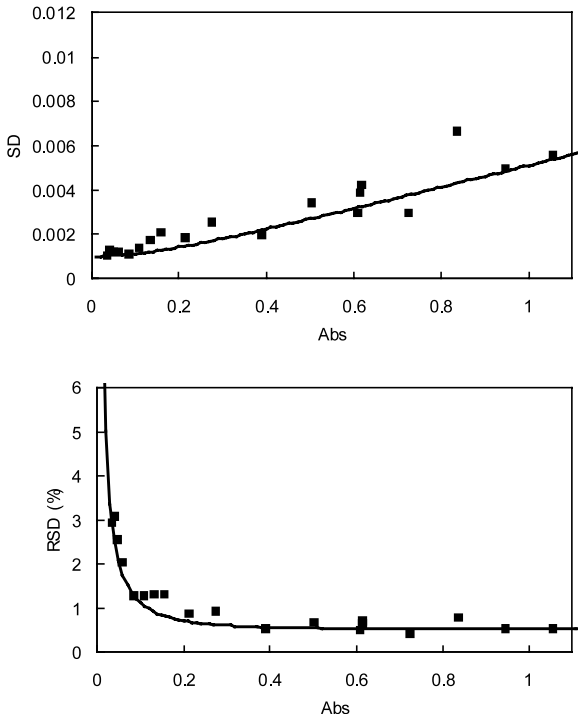

Fig. 4. Uncertainty of mixing of PNA and BSA solutions. Fifty microliter of PNA solution is pipetted into each well of a microplate and 50 μl of BSA solution is added to the well by a dispenser. The S.D. means the standard deviation of the averages of the absorbance data (121 points a well) for different wells. The R.S.D. is the S.D. divided by the mean of the averages. Each point (■) results from the data of 12 wells ($n=12$). Theoretical lines (___) are based on Eqs. (6) and (7).

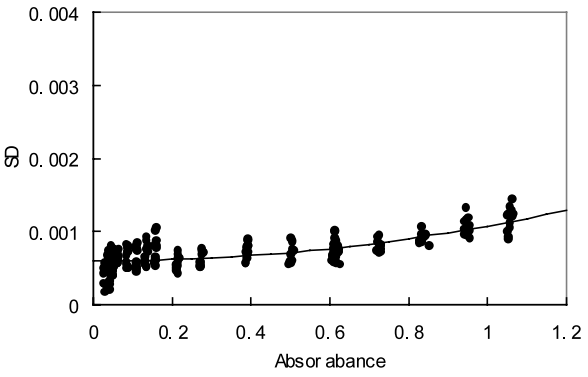

Fig. 5. Dependence of detector noise on absorbance. The same data as in Fig. 4 are used. Each S.D. value (●) is obtained from 121 data points of a well. Note that each S.D. value is obtained from the data of a well in Fig. 5. The fitted line is: $y=0.0007x^2-0.0002x+0.0006$.

Table 1
Volume error of pipette (50 μl)

	Distilled water	BSA
Average (μg)	51.1	51.0
S.D. (μg)	0.247	0.225
R.S.D. (%)	0.482	0.440

4. Results

Fig. 1 illustrates the time courses of the PNA production in the endotoxin assay. The slope of the enzyme reaction increases with increasing concentration. The bottom line corresponds to the blank and slightly increases with time.

The experimental precision or uncertainty referred to hereafter is the R.S.D. of the slopes of the reaction curves. The slope is represented by a straight line that is a least squares fit to a part of the reaction curve shown in Fig. 1. Fig. 2 shows the regions (20–25 min) used for the straight line fitting. In this study, the straight blank line (least squares fit) is subtracted from the actual curves to make the calibration line pass the origin. A primary demand for the success of our uncertainty prediction is a straight region of the reaction curves aside from the detector noise, since the error of the straight-line fitting should theoretically be predicted (see Appendix B).

Before considering the uncertainty of assay, we should examine the uniformity of light detection among the wells of the plate. If the detectability was different from well to well, it would be impossible to express the uncertainty of the assay by Eq. (5). Fig. 3 shows the average and S.D. of 121 data a well. The average concerns the volume of the solution pipetted into each well. The between-well scattering of the averages (see the upper figure of Fig. 3) is $\approx 1\%$ R.S.D. and does not greatly exceed the volume error of the pipette. The accuracy of the plate seems satisfactory.

The lower figure of Fig. 3 denotes the S.D. of the noise appearing on the detector output of a well. The noise S.D. is not greatly different from well to well and the precision is acceptable for every well. According to our experience, 4-fold difference in S.D. values between the wells has been found in a poorly adjusted detection unit.

Fig. 4 shows the statistics of the absorbances when 50 μl PNA and 50 μl BSA (20 mg/ml) are pipetted and mixed in the wells. The latter has no absorbance at 405 nm. The uncertainty of the mixing experiments varies depending on absorbance, as shown in Fig. 4 and Eqs. (1) and (2). The aim of this series of experiments is to predict the S.D. of mixture absorbances between the wells in the microplate.

The uncertainty prediction (Eqs. (1) and (2)) needs the S.D., S_W, of the inter-well absorbances and R.S.D., R_V, of the volume error of the pipette. The S.D., S_W, of the intra-well absorbances with the blank samples (distilled water) was observed to be 0.001 (average is 0.028). The pipette volume error was determined from gravimetric data ($R_V = 0.005$; also see Table 1). The S.D. and R.S.D. of absorbances take the form:

$$(dA)^2 = (0.001)^2 + (0.005)^2 A^2 \quad (6)$$

$$\left(\frac{dA}{A}\right)^2 = \left(\frac{0.001}{A}\right)^2 + (0.005)^2 \quad (7)$$

The theory and experiments are in good agreement over the absorbance range examined, as shown in Fig. 4. We should note that the theoretical lines (__) are not those fitted to the experimental results (■), but are derived from the data (e.g., Table 1) which are collected independently of the repeated experiments (■).

Four quantities are necessary for the equation of the uncertainty prediction (Eq. (5)): R_V, $\bar{a}$, $\Sigma_{i=1}^{N}(X_i - \bar{X})^2$, $\tilde{e}$. The R.S.D. of the pipette volume error has already been examined: $R_V = 0.005$. The calibration line, $a = 0.0398C_1 + 0.00007$, can be substituted for the mean of the slopes, $\bar{a}$. The term, $\Sigma_{i=1}^{N}(X_i - \bar{X})^2$, is constant, as long as the same data points (time) are used to obtain the slopes of the reaction curves by the least squares fitting, as shown in Fig. 2. In this study, $\Sigma_{i=1}^{N}(X_i - \bar{X})^2 = 770$.

Fig. 5 demonstrates the method for estimating the S.D. of the detector noise, $\tilde{e}$. The dependence of the noise on absorbance has been studies in detail by Ingle and Crouch [22,26]. The linear least squares fitting gives the relationship between the noise S.D., $\tilde{e}_i$ and absorbance, Y_i: $\tilde{e}_i = 0.0007Y_i^2 - 0.0002Y_i + 0.0006$. The relationship proposed by Ingle and Crouch is similar to that of Fig. 5 in the absorbance region up to 1.0. The absorbance examined here is below 0.2 and the invariant noise ($\tilde{e}_i = \tilde{e}$) can be assumed as: $\tilde{e} = 0.0006$, where the absorbance, Y_i, is fixed at 0.1.

From the above discussion, it follows that:

$$(R_1)^2 = 5 \times (0.005)^2 + \frac{(0.0006)^2}{770} \times \frac{1}{(0.0398C_1 + 0.00007)^2} \quad (8)$$

The uncertainty of the endotoxin assay can be described as a function of analyte concentration, C_1.

Fig. 6 shows the comparison between the R.S.D. from the repeated experiments (●; $n = 12$) and theoretical R.S.D. (—) estimated by Eq. (8). The theory is in good agreement with the practice. Again, we stress that the theoretical prediction is performed independently of the repeated experiments and that the theoretical line (—) of Fig. 6 is not a least squares fitting.

5. Discussion

The uncertainty of the endotoxin assay can successfully be estimated from the following quantities:

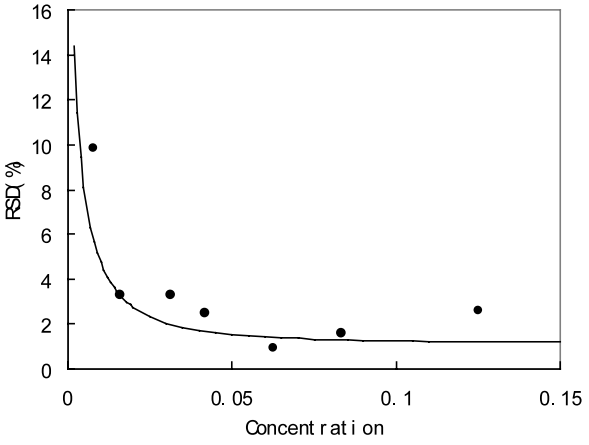

Fig. 6. Precision plot of endotoxin assay. ●, R.S.D. from repeated experiments ($n = 12$); —, theoretical R.S.D. estimated by Eq. (8).

1) volume error by pipette or dispenser;
2) detector noise.

The assay includes the mixing of the sample and LAL solutions, heating, detection and least squares fitting. The effect of the other factors such as temperature is not included in the uncertainty equation (Eq. (8)), but this effect can be regarded as being negligibly small.

The most advantageous feature of this paper is that the measurement R.S.D. in the endotoxin assay can be estimated from the separate experiments, rather than the endotoxin assay itself. Therefore, the repeated experiments can be dispensed with and the method of this paper will also be favorable from the viewpoints of economy and environmental sciences.

Appendix A: Derivation of Eq. (1) and Eq. (2)

The pathlength of absorption is invariable in the usual spectrophotometers, but it is changeable in the plate reader, since the light passes the absorbing solution in the vertical direction. If twice volume of absorbing solution is taken into a well, the absorbance becomes twice. On the other hand, the addition volume of not-absorbing solution exerts no influence on the absorbance measurement. If any volume of the not-absorbing solution is added to the absorbing solution in the well, the resulting absorbance is equal to the absorbance of the latter alone in the well.

Considering affecting Eq. (1) and Eq. (2) in the text, we can describe the absorbance measurement, A, as $A = W + kV$, where k is a coefficient to transform volume into absorbance. By differentiating the above equation with respect to W and V and squaring it, we can obtain $(dA)^2 = (dW)^2 + k^2(dV)^2$, where the non-correlation between W and V is taken into account (the mean of $dWdV$ is zero). If $A >> W$, then $k = (A - W)/V \approx A/V$. Using this approximation and writing the differentials in terms of S_X, we can obtain Eq. (1). Dividing Eq. (1) by A^2, we get Eq. (2). The tapered lateral faces of the microplate wells are neglected in the above derivation.

Appendix B: Derivation of Eq. (5)

The PNA production rate, r, is simplified as a second-order reaction with respect to the concentrations of the endotoxin, C_1 and LAL, C_2: $r = kC_1C_2$, where k is a coefficient. By differentiating the above equation with respect to C_1 and C_2 and dividing both sides by the reaction rate, r, we obtain:

$$\left(\frac{dr}{r}\right)^2 = \left(\frac{dC_1}{C_1}\right) + \left(\frac{dC_2}{C_2}\right)^2 \tag{B1}$$

where the influence of temperature and other environmental factors on coefficient, k, is assumed to be negligible ($dk = 0$).

The slope measurements depend on not only the concentrations as shown in Eq. (B1), but also the observed absorbance in the well reader. For example, the concentrations, C_1 and C_2, are kept constant, even if the volumes of both the endotoxin and LAL solutions to be mixed are doubled in a well. In this situation, however, the pathlength of absorption is doubled and the value of the slope measurement is also doubled.

The reaction indicator (PNA) is contained in the LAL solution and not in the analyte solution. Let V_2 be the right volume of the LAL solution in the well and δV_2 be the error of volume V_2. In the experiments of this paper, the same volumes of the endotoxin and LAL solutions are assumed to be taken and the final volume of the mixture is $2V_2$. Then, the rate, ΔC_2, of the concentration change caused by the mixing can be written as:

$$\Delta C_2 = \frac{\delta V_2}{2V_2} \tag{B2}$$

(a more strict derivation is given in Appendix C). On the other hand, the observed absorbance is independent of the volume of the analyte solution (see below). Therefore, the rate, ΔA, of the apparent absorbance change is $\Delta A = ((\delta V_2)/V_2)$. A volume error, δV_2, if positive, always leads to the increases in the concentration, ΔC_2 and absorbance, ΔA. The ratio of the concentration change to absorbance change is given: $\Delta A/\Delta C_2 = 2$. This equation indicates that the absorbance change is twice the concentration change. There-

fore, the S.D., dC_2, of the LAL concentrations should be tripled ($=1+2$) to describe the uncertainty of the slope measurements. Eq. (B1) can be re-written as:

$$(R_r)^2 = \left(\frac{dC_1}{C_1}\right)^2 + 9\left(\frac{dC_2}{C_2}\right)^2 \tag{B3}$$

which denotes the squared R.S.D. of the slope measurements without detection noise. Eq. (B3) is different from Eq. (B1) in that Eq. (B3) covers not only the change in the reaction rate, but also the observed absorbance change.

The R.S.D., dC_i/C_i, of the final concentrations of the mixture takes the form:

$$\frac{dC_1}{C_1} = \frac{V_2}{V_1+V_2}\left[\left(\frac{dV_1}{V_1}\right) + \left(\frac{dV_2}{V_2}\right)^2\right]^{1/2} \tag{B4}$$

$$\frac{dC_2}{C_2} = \frac{V_1}{V_1+V_2}\left[\left(\frac{dV_1}{V_1}\right) + \left(\frac{dV_2}{V_2}\right)^2\right]^{1/2} \tag{B5}$$

(see Appendix D). In the present study, $V_1 = V_2(=V)$, then $\frac{dC_1}{C_1} = \frac{dC_2}{C_2} = \frac{1}{\sqrt{\sqrt{2}}}\frac{dV}{V}$. By substituting this equation for Eq. (B3), we can obtain

$$(R_r)^2 = 5\left(\frac{dV}{V}\right)^2 \tag{B6}$$

The volume errors, dV_1/V_1 and dV_2/V_2, are assumed to be the same.

Let the absorbance observed at time or data point, X_i, be Y_i. As is well-known, the least square fitting of a straight line, $Y=aX+b$, to the noisy data gives the slope, a,

$$a = \frac{\sum_{i=1}^{N}(X_i - \bar{X})Y_i}{S_{XX}} \tag{B7}$$

where

$$\bar{X} = \frac{1}{N}\sum_{i=1}^{N} X_i, \quad S_{XX}\sum_{i=1}^{N}(X_i - \bar{X})^2$$

and N means the number of data points used for the least squares fitting. The variance, $\tilde{a}^2$, of the slopes is written as:

$$\tilde{a}^2 = \frac{1}{S_{XX}^2}\left\{\sum_{i=1}^{N}(X_i - \bar{X})^2\tilde{e}_i^2\right\} \tag{B8}$$

where $\tilde{e}_i$ denotes the S.D. of the noise at X_i. By dividing Eq. (B8) by the square mean, $\bar{a}_2$, of the slopes, we obtain:

$$(R_l)^2 = \frac{\left\{\sum_{i=1}^{N}(X_i - \bar{X})^2\tilde{e}_i^2\right\}}{\left(\sum_{i=1}^{N}(X_i - \bar{X})^2\right)^2} \times \frac{1}{\bar{a}^2} \tag{B9}$$

The objective equation for the uncertainty of the endotoxin assay is:

$$(R_T)^2 = 5\left(\frac{dV}{V}\right)^2 + \frac{\left\{\sum_{i=1}^{N}(X_i - \bar{X})^2\tilde{e}_i^2\right\}}{\left(\sum_{i=1}^{N}(X_i - \bar{X})^2\right)^2} \times \frac{1}{\bar{a}^2} \tag{B10}$$

where Eqs. (B6) and (B9) are substituted for Eq. (4). If the detector noise is constant irrespective of absorbance and $\tilde{e}_i = \tilde{e}$, $\tilde{a}^2 = \tilde{e}^2/S_{XX}$ (homoscedastic situation). In this situation ($\tilde{e}_i = \tilde{e}$), the objective equation takes the form of Eq. (5).

Appendix C: Derivation of Eq. (B2)

If a volume, V_2, of solution 2 is taken by a pipette and diluted with a volume, V_1, of solution 1, then the final concentration, C_2, is obtained from the initial concentration of solution 2, C_{20}: $C_2 = (V_2/(V_1+V_2))C_{20}$. The differential coefficient of the above equation with respect to V_2 is $((dC_2/dV_2) = ((V_1+V_2) - V_2)/((V_1+V_2)^2)\ C_{20} = V_1/(V_1+V_2)^2\ C_{20}$. Dividing the above by C_2, we can obtain $(dC_2/C_2) = V_1/(V_2(V_1+V_2))\,dV_2$. If $V_1 = V_2$ and $\Delta C_2 = dC_2/C_2$ we can obtain Eq. (B2).

Appendix D: Derivation of Eq. (B4) and Eq. (B5)

Let the initial and final concentrations of solution 1 be C_{10} and C_1. After the dilution with

another solution of volume, V_2, the final concentration, C_1, of solution 1 is given as $C_1 = V_1/(V_1 + V_2)\ C_{10}$. The total differential is

$$dC_1 = \frac{V_2}{(V_1 + V_2)^2}\ C_{10}\ dV_1 - \frac{V_1}{(V_1 + V_2)^2}\ C_{20}\ dV_2$$

The independent randomness of differentials, dV_1 and dV_2, leads to

$$(dC_1)^2 = \frac{V_2^2}{(V_1 + V_2)^4}\ C_{10}^2\ (dV_1)^2 + \frac{V_1^2}{(V_1 + V_2)^4}\ C_{10}^2\ (dV_2)^2$$

Dividing the above equation by the right concentration, C_1, we can obtain

$$\left(\frac{dC_1}{C_1}\right)^2 = \frac{V_2^2}{(V_1 + V_2)^2}\ \left[\left(\frac{dV_1}{V_1}\right)^2 + \left(\frac{dV_2}{V_2}\right)^2\right]$$

The square root of the above leads to Eq. (B4). The equivalent equation, but differently derived, has already been published [27].

References

[1] Y. Yoshimi, M. Yotsumoto, K. Sakai, ASAIO J. 41 (1995) 68–71.
[2] D. Petysch, W.D. Deckwer, F.B. Anspach, Anal. Biochem. 259 (1998).
[3] R.N. Berzofsky, ALTEX 12 (1995) 93–97.
[4] C. Yamamoto, Rinsho Byori 45 (1997) 1167–1171.
[5] M. Liebsch, ALTEX 12 (1995) 76–80.
[6] The Pharmacopoeia of Japan, The Ministry of Health and Welfare, Tokyo, 1996.
[7] British Pharmacopoeia, Her Majesty's Stationery Office, London.
[8] The United States Pharmacopoeia, 2002.
[9] Analytical Methods Committee, Analyst 120 (1995) 2303–2308.
[10] C. Th, J. Alkemade, W. Snelleman, G.D. Boutilier, B.D. Pollard, J.D. Winefordner, T.L. Chester, N. Omenetto, Spectrochim. Acta 33B (1978) 383–399.
[11] G.D. Boutilier, B.D. Pollard, J.D. Winefordner, T.L. Chester, N. Omenetto, Spectrochim. Acta 33B (1978) 401–415.
[12] P.W.J.M. Boumans, Spectrochim. Acta 46B (1991) 917–939.
[13] H.C. Smit, H.L. Walg, Chromatographia 9 (1976) 483–489.
[14] T.J. Bahowick, R.E. Synovec, Anal. Chem. 67 (1995) 631–640.
[15] Y. Hayashi, R. Matsuda, Anal. Chem. 66 (1994) 2874–2881.
[16] R. Matsuda, Y. Hayashi, S. Sasaki, K. Saito, K. Iwaki, H. Harakawa, M. Satoh, Y. Ishizuki, T. Kato, Anal. Chem. 70 (1998) 319–327.
[17] E.D. Prudnikov, Fresenius J. Anal. Chem. 337 (1990) 412–415.
[18] P.W.J.M. Boumans, Anal. Chem. A66 (1994) 459A–467A.
[19] Grushka, I. Zamir, Chemical Analysis (1989) 529–561.
[20] N.W. Bower, J.D. Inglejr, Jr, Anal. Chem. 48 (1976) 686–692.
[21] N.W. Bower, J.D. Inglejr, Anal. Chem. 49 (1977) 574–579.
[22] J.D. Ingle, Jr, S.R. Crouch, Spectrochemical Analysis, Prentice-Hall, Englewood Cliffs, NJ, 1988.
[23] R.A. Day, A.L. Underwood, Quantitative Analysis, Prentice Hall, Englewood Cliffs, NJ, 1991.
[24] H.G. Hecht, Mathematics in Chemistry, Prentice Hall, Englewood Cliffs, NJ, 1990.
[25] Y. Hayashi, R. Matsuda, R.B. Poe, Analyst 121 (1995) 591–599.
[26] J.D. Ingle, Jr, S.R. Crouch, Anal. Chem. 44 (1972) 1375–1386.
[27] Y. Hayashi, R. Matsuda, Anal. Sci. 10 (1994) 881–888.