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Abstract

In immunoassay, calibration curves are used for transformation of sample responses into concentrations. The calibration curve is a linear or nonlinear regression curve fitted to a set of calibrators (standards) with known concentrations. In this report we discuss consequences of random errors in the dispensed volumes of the calibrators. Provided that these errors are the main source of intra-assay variation, a constant coefficient of variation in concentration could be expected. It is suggested that the curve parameters in this case be estimated by inverse regression (i.e. regression of X on Y), weighted by squared calibrator concentrations. An approximate formula is derived for the standard deviation in response, given that the response follows a four-parameter logistic function.

Keywords: Calibration, coefficient of variation, four-parameter logistic function, immunoassay, inverse regression

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1 Introduction

The immunoassay is an analytical technique for measuring sample components, using antibodies or antibody-related reagents (Hage, 1999). The response of the immunoassay, usually radiation or luminescence, is assumed to be functionally related to the concentration of the sample component. The function could be a straight line or some other linear or monotonic nonlinear function. It is necessary to establish the relationship between concentration and response. This is done by measuring samples with known concentrations, called calibrators or standards. A calibration curve is fitted to the responses of the calibrators. The fitted curve is used for inverse prediction when responses of unknowns (i.e. samples with unknown concentrations) are transformed into estimates of concentration through the calibration curve.

There are many possible sources of errors in immunoassays (Jones et al., 1995). For example, differences between days and laboratories in temperatures or reagents give rise to inter-assay variation. Other sources, such as differences in timing, washing and signal detection, cause intra-assay variation. Many components of intra-assay variation are minimized in automated measuring instruments. Differences in volumes of the sample (e.g. blood serum), dispensed by a pipette, remain one of the most important reasons for intra-assay variation. In highly automated systems, the small random variation in the volumes of the samples could be the main source of random variation in predicted concentrations. We shall consider the consequences of this presumption.

In Section 2, we show that if the calibrators are dispensed with normally distributed volumes, concentrations could be regarded as normally distributed with constant coefficient of variation. In Section 3 we consider consequences for fitting calibration curves. It is shown that the assumption of normally distributed volumes suggests inverse regression, as originally proposed by Krutchkoff (1967) for linear calibration. According to this approach, concentrations are regressed on responses, rather than responses on concentrations as in classical regression. In our application the calibration curve can be nonlinear.

The research on inverse regression for calibration was reviewed by Osborne (1991). The inverse estimator of sample concentrations is biased and inconsistent, but may, within the calibration range, give smaller mean square error than the classical estimator. This was confirmed by Tellinghuisen (2000), who especially investigated small calibration data sets. The properties of inverse regression is usually studied under the assumptions of random calibrator responses and known concentrations. In this report we arrive at inverse regression based on assumptions of random numbers of molecules and known

responses. Tellinghuisen (2000) pointed out that modern spectrophotometers can give responses with precision that "exceeds that of the 'known' concentrations of calibration samples in much routine work, making regression of concentration upon absorbance fully legitimate." This is the starting-point of our investigation.

We propose inverse regression with weights inversely proportional to squared calibrator concentrations. We call this method weighted inverse regression, as distinct from weighted classical regression. Weights are needed in calibration of immunoassays in order to improve predictions at low concentrations (Carroll, 2003). In weighted classical regression, the variance in response is often assumed to be an increasing function of the response level. Specifically the variance in response is often modeled as a power function of the expected response, that is

$$\mathrm{var}(Y) = \phi\,\mathrm{E}(Y)^{\theta}. \tag{1}$$

A parameterized variance function such as (1) is needed, because when the number of replicates is small, as is usually the case in immunoassays, it is inefficient to use sample variances as weights (Carroll and Cline, 1988). Methods for estimation of variance parameters have been proposed by Rodbard et al. (1976), Raab (1981), Davidian and Carroll (1987), Davidian (1990), Goos et al. (2001) and Sadler (2002). The weighted inverse regression that we propose does not require variance parameters.

In Section 4 we investigate consequences on the variance in response when the relationship between response and concentration is described by the four-parameter logistic function. This function is often used for calibration in analytical procedures (O'Connell et al. 1993). An example is given in Section 5.

2 Constant CV

Let ξ_i and y_i, $i = 1, 2, \ldots, n$, denote the true concentrations and the observed responses of the calibrators, respectively. Let V_i denote the dispensed volume of the i:th calibrator. We shall assume that the volume is a normally distributed random variable with the same expected value and variance for all calibrators, i.e. V_i is $\mathrm{N}(\mu, \sigma^2)$, $i = 1, 2, \ldots, n$. We shall also assume that the expected volume μ is known.

The response y_i is functionally related to the number of molecules N_i of the particular substance, that is $y_i = g(N_i)$, $i = 1, 2, \ldots, n$. In many applications it could be assumed that the response is proportional to the number of molecules, but often a linear or nonlinear function, for example describing a logistic growth, is more realistic. The calibration curve is usually written

as a function f from concentration to response. Given the basic functional relationship g, the calibration curve function f is easily obtained by defining X_i as N_i/μ, because then $f(X_i) = g(X_i\mu)$.

Because $N_i = \xi_i V_i$ it is clear that N_i is $\mathrm{N}(\xi_i\mu, \xi_i^2\sigma^2)$. Thus $y_i = g(\xi_i\mu + E_i)$, where E_i is $\mathrm{N}(0, \xi_i^2\sigma^2)$. Then $y_i = f(\xi_i + E_i/\mu)$, and $\xi_i = f^{-1}(y_i) + e_i$, where $e_i = -E_i/\mu$. Let γ denote the coefficient of variation in the dispensed volume, that is $\gamma = \sigma/\mu$. Then e_i is $\mathrm{N}(0, \xi_i^2\gamma^2)$. This suggests weighted inverse regression, as we shall see in Section 3.

However, even if the volumes are always dispensed without error the number of molecules vary between samples. The number of molecules could be Poisson distributed, conditioned on the volume. To study this argument, assume that N_i, given V_i, is Poisson$(V_i\xi_i)$, where V_i is $\mathrm{N}(\mu, \sigma^2)$, $i = 1, 2, \ldots, n$. Because the number of molecules is very large, usually larger than 1 million, the conditional distribution of N_i is approximately $\mathrm{N}(V_i\xi_i, V_i\xi_i)$. Then

$$\mathrm{E}(N_i) = \mathrm{E}(\mathrm{E}(N_i \mid V_i)) = \mathrm{E}(V_i\xi_i) = \xi_i\mu, \tag{2}$$

$$\mathrm{var}(N_i) = \mathrm{E}(\mathrm{var}(N_i \mid V_i)) + \mathrm{var}(\mathrm{E}(N_i \mid V_i)) = \xi_i\mu + \xi_i^2\sigma^2. \tag{3}$$

The coefficient of variation in X_i, defined as $X_i = N_i/\mu$, equals the coefficient of variation in N_i, which by (2) and (3) can be written

$$\sqrt{\gamma^2 + \frac{1}{\mathrm{E}(N_i)}}. \tag{4}$$

For a Poisson distributed random variable U, the squared coefficient of variation is $1/\mathrm{E}(U)$. Thus, the total coefficient of variation (4) in X_i is the square root of a sum of two variation components: the squared coefficient of variation in the sample volume, and the squared coefficient of variation in the Poisson distribution. When N_i is large, (4) approximately equals γ.

3 Curve fitting

Based on the results of Section 2, we now consider fitting of calibration curves under the assumption that the response y_i is a function f of the concentration X_i where X_i is $\mathrm{N}(\xi_i, \xi_i^2\gamma^2)$, $i = 1, 2, \ldots, n$. The function f is linear or nonlinear in k parameters $\beta_1, \beta_2, \ldots, \beta_k$ that should be estimated.

The probability density function for X_i, given the response y_i, can be written

$$\frac{1}{\sqrt{2\pi\gamma^2\xi_i^2}}\ \exp\left(\frac{(f^{-1}(y_i) - \xi_i)^2}{2\gamma^2\xi_i^2}\right),\ i = 1, 2, \ldots, n,$$

and the logarithm of the likelihood equals

$$\sum_{i=1}^{n}\left(\frac{1}{2}\log(2\pi\gamma^2\xi_i^2) - \frac{(f^{-1}(y_i)-\xi_i)^2}{2\gamma^2\xi_i^2}\right). \tag{5}$$

Let $\boldsymbol{\beta} = (\beta_1, \beta_2, \ldots, \beta_k)'$, $\boldsymbol{\xi} = (\xi_1, \xi_2, \ldots, \xi_n)'$, $\mathbf{y} = (y_1, y_2, \ldots, y_n)'$ and $\mathbf{f}^{-1}(\mathbf{y}) = (f^{-1}(y_1),\ f^{-1}(y_2), \ldots, f^{-1}(y_n))'$. Then (5) is maximized over $\boldsymbol{\beta}$ by minimizing

$$(\mathbf{f}^{-1}(\mathbf{y}) - \boldsymbol{\xi})'\mathbf{D}^{-1}(\mathbf{f}^{-1}(\mathbf{y}) - \boldsymbol{\xi}), \tag{6}$$

where $\mathbf{D} = \gamma^2\text{diag}\{\xi_1^2, \xi_2^2, \ldots, \xi_n^2\}$. The maximum likelihood estimator of $\boldsymbol{\beta}$ is the generalized least squares estimator, that is the estimator that minimizes (6). Under regularity conditions (Seber and Wild, 1989), the generalized least squares estimator is asymptotically $\text{N}(\boldsymbol{\beta}, \mathbf{V})$, where

$$\mathbf{V} = \left(\frac{d\mathbf{f}^{-1}}{d\boldsymbol{\beta}'}\right)'\mathbf{D}^{-1}\frac{d\mathbf{f}^{-1}}{d\boldsymbol{\beta}'}.$$

4 Variance in the four-parameter logistic function

Let y denote the immunoassay response, which is a monotone increasing function of the concentration x. Let β_1 denote the response at zero concentration, and let β_2 denote the limit response at an infinitely high concentration. Define the proportion p as

$$p = \frac{y-\beta_1}{\beta_2-\beta_1}. \tag{7}$$

This proportion p is in immunoassay often assumed to follow the logistic growth model

$$\frac{dp}{dt} = \beta_4\ p\ (1-p), \tag{8}$$

where $t = \log(x)$ and β_4 is a constant that defines the growth rate. The solution to the differential equation (8) can be written

$$p = \frac{1}{1+\exp^{-(\alpha+\beta_4 t)}} = \frac{1}{1+(\beta_3/x)^{\beta_4}}, \tag{9}$$

where $\alpha = -\beta_4\log(\beta_3)$ is an arbitrary constant. By (7) and (9), the response y, as a function of the concentration x, is

$$y(x) = \beta_2 + \frac{\beta_1-\beta_2}{1+(x/\beta_3)^{\beta_4}}, \tag{10}$$

which is the four-parameter logistic function. In (10), β_3 denotes the concentration that gives the response $(\beta_1 + \beta_2)/2$. Now, assume that x is a random variable. Then y is a random variable according to the model (10). If we let

$$z = \frac{x - \mathrm{E}(x)}{\mathrm{E}(x)}, \tag{11}$$

we can write (10) as

$$y(x) = \beta_2 + \frac{\beta_1 - \beta_2}{1 + (\mathrm{E}(x)/\beta_3)^{\beta_4}(z+1)^{\beta_4}}. \tag{12}$$

When the coefficient of variation is small, large values of z are unlikely. In order to approximate the variance of (10), we expand (12) about $z = 0$, which yields

$$y(x) = \beta_2 + \frac{\beta_1 - \beta_2}{1 + (\mathrm{E}(x)/\beta_3)^{\beta_4}} - \frac{(\beta_1 - \beta_2)\beta_4(\mathrm{E}(x))^{\beta_4} z}{(1 + (\mathrm{E}(x)/\beta_3)^{\beta_4})^2 \beta_3^{\beta_4}} + O(z^2), \tag{13}$$

with z as in (11). Provided that the variance due to the last term is negligible, by (10), (11) and (13),

$$\mathrm{var}(y(x)) \approx \left(\left. \frac{dy}{dx} \right|_{x=\mathrm{E}(x)} \right)^2 \mathrm{var}(x).$$

Consequently, the standard deviation σ_y in response approximately equals the standard deviation σ_x in concentration multiplied by the slope of the curve at $E(x)$:

$$\sigma_y \approx \frac{dy}{dx}\, \sigma_x = \frac{dy}{dp}\frac{dp}{dt}\frac{dt}{dx}\, \sigma_x = \frac{\beta_4(\mu_y - \beta_1)(\beta_2 - \mu_y)\gamma_x}{(\beta_2 - \beta_1)}\ , \tag{14}$$

where γ_x is the coefficient of variation, i.e. $\gamma_x = \sigma_x / E(x)$. Equation (14) is an approximate formula for the standard deviation, as a function of the response level μ_y, and it could be compared to other variance functions, such as (1). According to (14), the standard deviation is approximately a second-order polynomial in the response level, provided a constant coefficient of variation in concentration.

5 Example: ImmunoCAP Specific IgG

ImmunoCAP Specific IgG (Phadia AB, Uppsala, Sweden) measures antigen-specific IgG antibodies in human serum and plasma. In this example we

consider a data set of responses y_{ijk}, $i = 1, 2, \ldots, N$, $j = 1, 2, \ldots, J$, $k = 1, 2, \ldots, n_{ij}$ of duplicate measurements (i.e. $n_{ij} = 2$) of $J = 6$ calibrators assayed in $N = 12$ runs. Let x_{ijk} denote the concentrations of the calibrators and note that $x_{ij1} = x_{ij2}$. The four-parameter logistic function was fitted, for each assay run, by minimizing (6) with the procedure `nlin` in SAS 9.1 (SAS Institute Inc., Cary, NC, USA). The fit of the first assay run is shown in Figure 1.

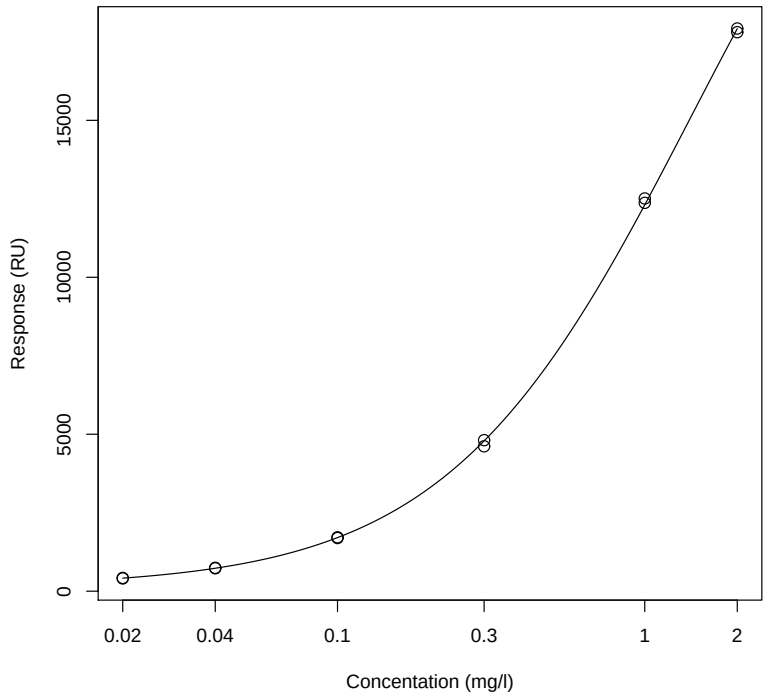

Figure 1: A four-parameter logistic curve fitted by weighted inverse regression to the calibration data of the first assay run.

Let $\hat{\beta}_{1i}$, $\hat{\beta}_{2i}$, $\hat{\beta}_{3i}$ and $\hat{\beta}_{4i}$ denote the estimates of β_1 β_2, β_3 and β_4, respectively, in assay run i, $i = 1, 2, \ldots, N$. The calibrator concentrations x_{ijk} were estimated by the inverse of the four-parameter logistic function, that is by

$$x_{ijk} = \hat{\beta}_{3i} \left(\frac{y - \hat{\beta}_{1i}}{\hat{\beta}_{2i} - y_{ijk}} \right)^{1/\hat{\beta}_{4i}}.$$

Table 1: Estimated coefficients of variation (CV)

Calibrator (mg/l)	CV (%)
0.02	2.53
0.04	1.83
0.10	1.62
0.30	2.07
1.00	1.99
2.00	2.01

Let c_{ij} denote the sample coefficient of variation in assay run i, for calibrator j. The common coefficients of variation in the duplicate measurements were, per calibrator, estimated by

$$\left(1 - \frac{1}{4\sum_{i=1}^{N}(n_{ij}-1)}\right)^{-1} \sqrt{\frac{\sum_{i=1}^{N}(n_{ij}-1)\, c_{ij}^2}{\sum_{i=1}^{N}(n_{ij}-1)}}\ ,\ j = 1, 2, \ldots, 5,$$

as suggested by Forkman (2008). The estimates are reported in Table 1.

The approximate F-test for equality of coefficients of variation, suggested by Forkman (2008), can be applied under assumption of independence. Let

$$u_{ij} = \frac{c_{ij}^2}{1 + c_{ij}^2(n_{ij}-1)/n_{ij}}\ , \qquad j = 1, 3;\ i = 1, 2, \ldots, N.$$

The F-statistic for the largest coefficient of variation (2.53%), compared to the smallest (1.62%), is

$$F_{max} = \frac{\sum_{i=1}^{N}(n_{i1}-1)u_{i1} / \sum_{i=1}^{N}(n_{i1}-1)}{\sum_{i=1}^{N}(n_{i3}-1)u_{i3} / \sum_{i=1}^{N}(n_{i3}-1)} = 2.43. \tag{15}$$

The 95:th percentile of the F-max distribution for 6 mean squares, all having 12 degrees of freedom is 5.72 (Nelson, 1987). The coefficients of variation, estimated as in Table 1, are not significantly different on level 5%.

The variance in response is often assumed to be a power function of the mean. The variance parameters ϕ and θ in (1) could be estimated by linear regression: $\log s_{ij}^2 = \log\phi\ +\ \theta \log \bar{y}_{ij}$, as proposed by Rodbard et al. (1976). Based on a regression of $12 \cdot 6 = 72$ log variances on average responses, ϕ and θ were estimated to $\hat{\phi} = 0.0026$ and $\hat{\theta} = 1.47$, respectively.

In Figure 2, weighted inverse regression is compared to weighted classical regression (i.e. weighted least squares using the estimates $\hat{\phi}$ and $\hat{\theta}$). In Figure 3,

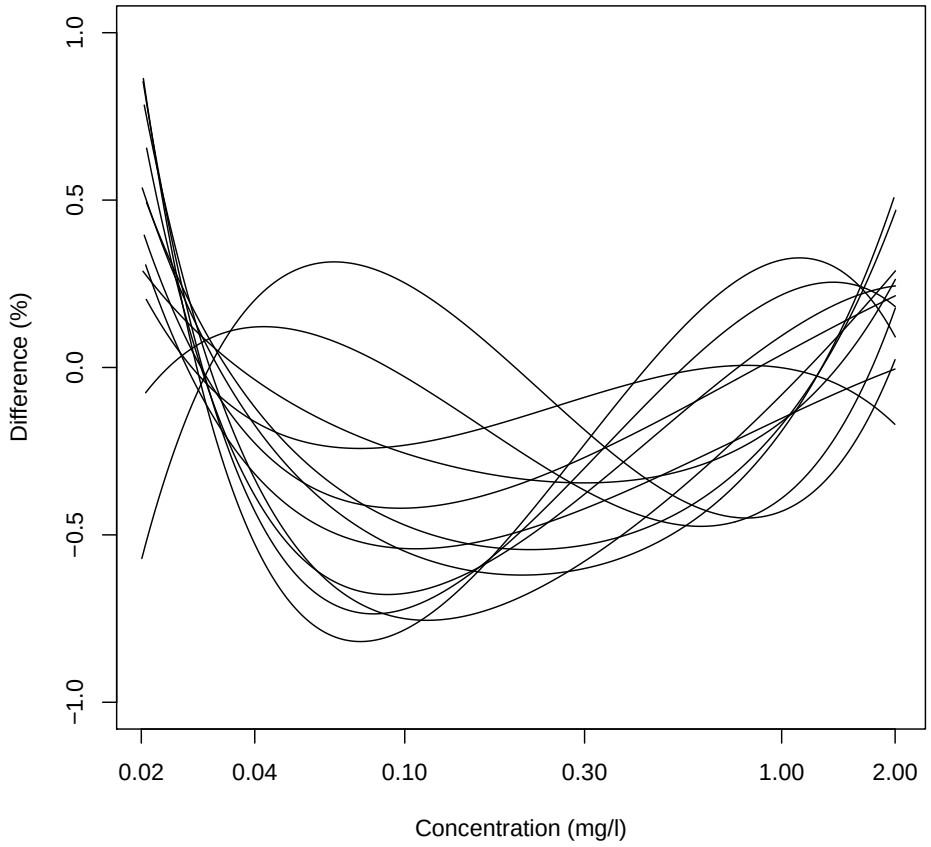

Figure 2: Weighted inverse regression (WIR) compared to weighted classical regression (WCR). Differences, on the y-axis, measured by log ratios of concentrations predicted by WIR to concentrations predicted by WCR. Concentrations, on the x-axis, predicted by WCR.

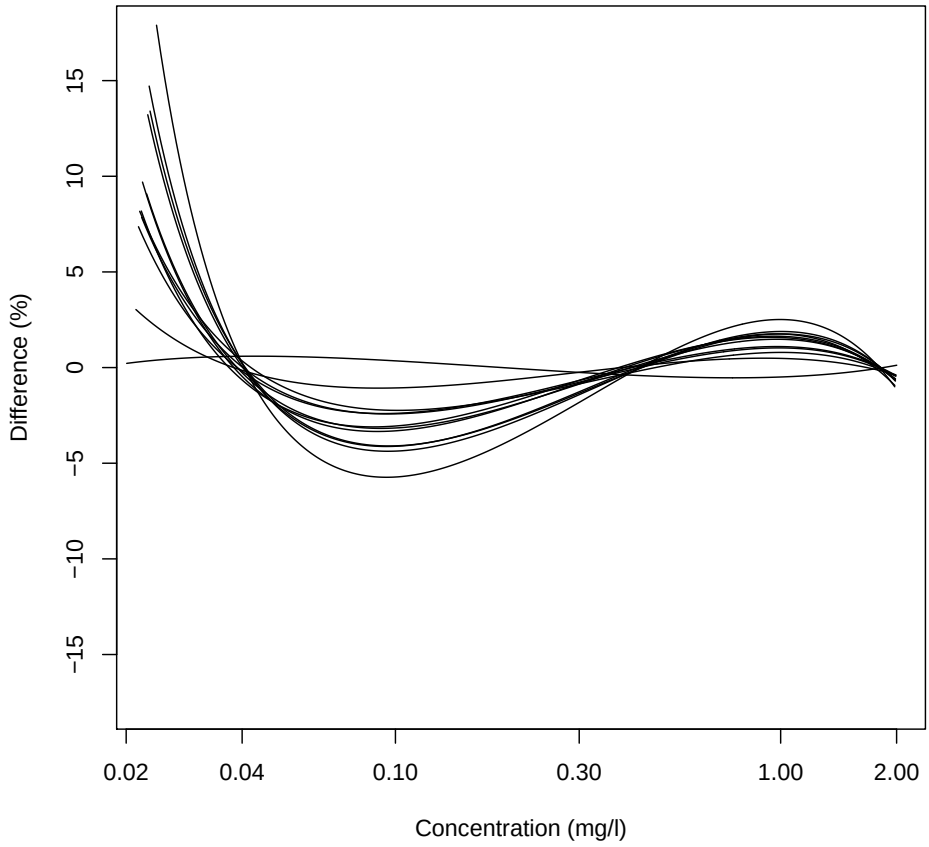

Figure 3: Weighted inverse regression (WIR) compared to classical regression without weights (CR). Differences measured by log ratios of concentrations predicted by WIR to concentrations predicted by CR. Concentrations, on the x-axis, predicted by CR.

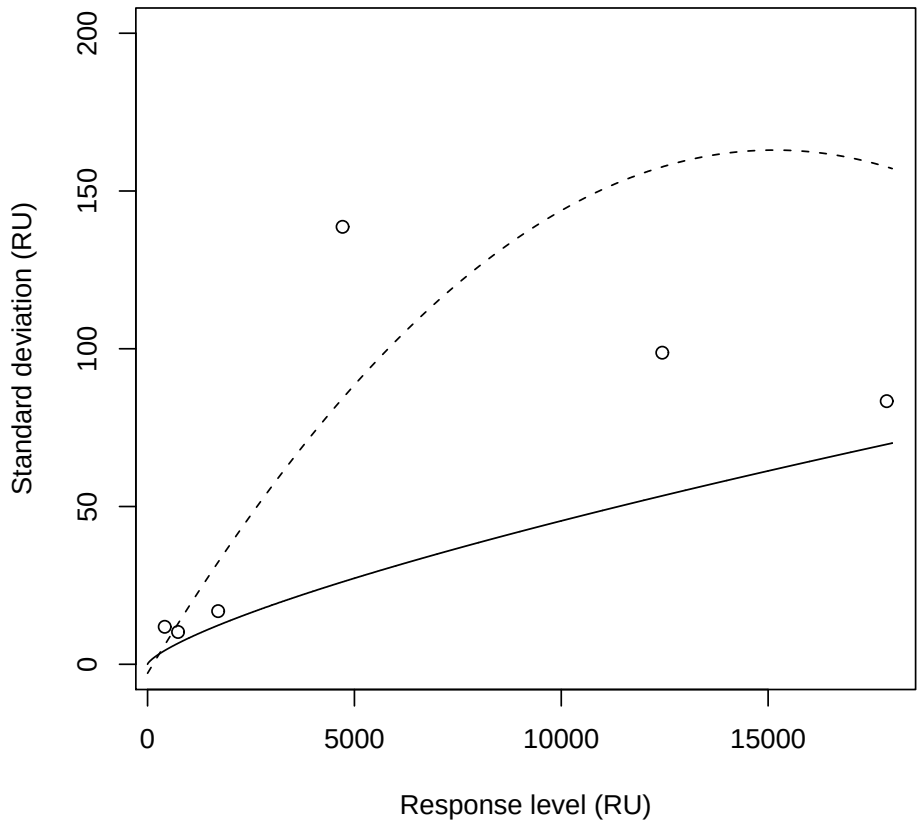

Figure 4: Comparison of variance functions in the first assay run. Circles: Standard deviations by means, per calibrator. Solid line: Standard deviation according to the power function (1), with $\phi = 0.0026$ and $\theta = 1.47$, by response level $E(y)$. Dashed line: Standard deviation according (14) by response level μ_y, with curve parameters estimated by weighted inverse regression.

weighted inverse regression is compared to classical regression without weights (i.e. ordinary least squares). Response values distributed uniformly on the response axis were transformed into concentration by three methods. The differences between the methods were measured by the logarithm of the ratio between the predicted concentrations and displayed on the y-axes of the figures as percentages.

Weighted inverse regression and weighted classical regression never differed more than 1% in predicted concentration (Figure 2). There could be systematic differences between the two methods, but these systematic differences do not appear to be large. The differences between weighted inverse regression and classical regression without weights were much larger, especially in the lower part of the measuring range. Four assay runs showed differences in predicted concentrations larger than 10% (Figure 3).

In Figure 4, the approximate formula for the variance in response (14) is compared to the power-of-the-mean function (1). The figure shows the standard deviations, on the y-axis, and the average responses, on the x-axis, for the calibrators in the first assay run. The solid line is the power function, with variance parameters estimated by the method proposed by Rodbard et al. (1976), based on the whole data set. In the first assay run, the standard deviations are larger than predicted by the solid line. The dashed line is the approximate formula (14), introduced in this report, with curve parameter estimates obtained by weighted inverse regression. The maximum response variance is obtained at response level 15,022 RU, which is the response corresponding to concentration β_3 in (10). This concentration is usually denoted ED50.

6 Discussion

In this report, we have studied calibration of immunoassays, under the assumption that the errors in the responses are small, and negligible, compared with the errors in the concentrations. This may be the case in automated testing systems with high precision. It is well known that inverse regression is appropriate in this situation (Lavagnini and Magno, 2007). When large errors are present in both response and concentration, methods for errors-in-variables modeling could be applied, with concentration considered as a controlled variable (Cheng and Van Ness, 1999).

We have assumed that the coefficient of variation is constant in concentration, which implies that the variation in response approaches zero as the concentration approaches zero. However, samples with zero concentration usually vary in response. This was recognized by Rocke and Lorenzato (1995), who

advocated estimation of two variance components: one giving approximately constant coefficient of variation in response for high levels of concentration, and one giving approximately constant standard deviation in response for very low levels of concentrations. We agree with this view. The assumption of a constant coefficient of variation is not realistic for samples and calibrators with very low concentrations. However, it could be reasonable within the measuring range, that is above the quantitation limit, where samples are "quantitatively determined with stated acceptable precision and trueness" (Clinical and Laboratory Standards Institute, 2004).

In analytical procedures, the coefficient of variation is the standard measure of dispersion. Methods are calibrated in order to reduce the variation in concentration. The coefficients of variation in concentration should be as small as possible, over the measuring range. Calibration by minimizing squared relative errors in concentration is in line of this reasoning.

The variance in concentration is often increasing with the level of concentration. In Section 2, a possible explanation of this phenomenon was given. As a consequence, the variance in response should increase with the level of response, until it gets constrained by the upper limit of the system. If the response could not exceed an upper limit β_2, as in the four-parameter logistic function (10), the variance in response should decrease as the response y approaches β_2. This mechanism is included in the approximate formula (14) for the standard deviation in response. According to (14), the standard deviation obtains its maximum value at $y = (\beta_2 - \beta_1)/2$, i.e. at the concentration $x = \beta_3$, which is commonly denoted ED50. In applications, it is often assumed that the variance is an increasing function of the response level, and specifically that the variance is a power function (1) of the expected response. For increasing calibration curves, this could be a good approximation below concentration ED50, where the upper response limit of the system does not affect the measurements.

Systems need to be calibrated when the relationship between response and concentration changes. However, not only the parameters of the calibration curve may change, but also the parameters of the variance function. Findlay et al. (2000) recommend that the variance parameters be estimated by pooling information from multiple runs, and that re-evaluation be made periodically during routine assay use. Different methods could give different estimates of the variance parameters. By weighted inverse regression, these problems need not be considered, because variance parameters are not included in the method. As illustrated by the example, concentrations predicted by weighted regression, inverse or classical, may differ noticeably from concentrations predicted by

regression without using weights. The method for weighting is usually less important. Weighted inverse regression could for these reasons be a convenient alternative to classical regression.

We have focused on calibration and prediction, rather than construction of confidence intervals. Methods for interval estimation based on single assay runs are likely to produce too small confidence sets, because they do not include inter-assay imprecision. The total variation of the analytical method should be taken into account when assessing predicted concentrations. This total variation can be estimated in a precision study including several assay runs performed under varying conditions, preferably at different laboratories. If possible, precision studies should include several measuring instruments and batches of reagents. The methods suggested by Johnson and Krishnamoorthy (1995) and Bhaumik and Gibbons (2005) could be used for constructing confidence intervals for unknown sample concentrations that have been measured at several laboratories. However, in applications samples with unknown concentrations are usually measured only once. Information from precision studies is required to assess the quality of the measurements.

Regardless the method for calibration we recommend that rules for outlier detection be defined, and control limits be set up, based on experience with the analytical procedure, so that erroneous responses are removed automatically before curve fitting.

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