Limits of Detection and Quantification, as Applied to an Assay for Digoxin

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The low-range sensitivity of a drug assay in routine use is an important aspect of that assay’s clinical usefulness. We define three concepts for determining low range sensitivity: \( L_C \), the detection threshold; \( L_0 \), the lower limit of “virtually ensured” detection; and \( L_Q \), the limit of quantitative determination. To illustrate these concepts, we apply them to a radioimmunoassay for digoxin, and show that for standards ranging from 0.3 to 5.0 \( \mu g/L \), the \( L_0 \) is 0.35 \( \mu g/L \) (±10%).

Additional Keyphrases: statistics  · radioimmunoassay

A recent survey conducted by the College of American Pathologists4 has underscored the confused state of affairs surrounding detection limits for analytical methods. Participants in the Therapeutic Drug Monitoring survey program were asked to list their “lowest amount detectable” for a variety of analytes. The responses varied over a surprisingly wide range. Most puzzling was the large number of respondents who stated that zero was their limit of detection.

The survey shows that the concepts of detection limits and related measurements need to be clarified. Procedures have been developed for such determinations in many other fields. In what follows we explain the concepts, then apply them to a data set. This is intended to be illustrative, not an instruction kit; the details of the statistical analysis must depend on the experimental design and the properties of the particular assay. However, we have tried to be specific enough that a statistically qualified person can follow and even re-apply the analysis we summarize here.

Because no analytical method has a true limit of zero, we became interested in trying to define the true limit of detectability, drawing on previously developed statistical methods (1,2) of basic importance for this report.

We chose as a practical example of the concept the radioimmunoassay (RIA) for digoxin, as described below. This analysis is widely used in various forms and is of considerable clinical importance.

Theory

This discussion follows the development of concepts described by Currie (1). Because of measurement error, a specimen with a particular true concentration of the analyte will produce various measured values, if several samples are prepared from it and assayed blindly (i.e., without knowledge of their common origin). A small fraction of measurement errors will be unusually large, and typically about half of them will be positive.

It follows that a zero-concentration specimen is very likely to look positive and has a small chance of being measured as quite positive. These considerations lead to setting a threshold value, \( L_C \), defining “detection”: if the measured value \( \hat{x} \) exceeds \( L_C \), then the presence of the analyte is “detected”;

otherwise it is not. If \( L_C \) is set high enough, then the zero specimens are nearly sure not to be detected, which is desirable. If enough information about assay values for zero specimens is available, then \( L_C \) can be set to provide a chosen (high) probability (e.g., 0.95) that a zero specimen will not be “detected.” This process is summarized in the following sections.

First Concept: Detection Threshold, \( L_C \)

Let \( \theta \) be the true concentration and \( \hat{x} \) be a measured value. We shall define the analyte as “detected” if \( \hat{x} \) exceeds \( L_C \). A value for \( L_C \) is chosen so that the probability of obtaining a value of \( \hat{x} > L_C \) if \( \theta = 0 \) is only 0.05, or some other desired small probability, \( a \). The smaller the value for \( a \) that is chosen, the larger must be \( L_C \). Note that \( L_C \) is value of \( \hat{x} \), the measurement.

Qualitative understanding of \( L_C \) is not difficult. If a specimen has \( \theta = 0 \), then, as described, there is only a small probability, \( a \), that the analyte will be “detected” in that specimen. If \( \theta \) is a little larger than zero, then the probability of detection is a little larger than \( a \), though still small. If a specimen had concentration \( \theta \) equal to \( L_C \), then symmetry of measurement errors would result in exactly an even chance of \( \hat{x} > L_C \) (and of \( \hat{x} < L_C \)). Thus, if \( \theta = L_C \), the probability of detection is exactly 0.5, if the measurement errors have a symmetric distribution. For larger values of \( \theta \), the probability of detection is larger. How large must \( \theta \) be for detection to be virtually ensured? This question can be answered if we quantify “virtually ensured.”

Second Concept: Lower Limit of “Virtually Ensured” Detection, \( L_Q \)

Let us choose a small probability \( \beta \) (such as 0.10 or 0.05 or 0.01) so that we are satisfied to regard 1 − \( \beta \) as “virtually ensured” detection. Then we define \( L_Q \) as a value of \( \theta \) (true specimen concentration), such that if \( \theta = L_Q \), the measured value \( \hat{x} \) has a probability of at least (1 − \( \beta \)) of exceeding \( L_C \), and so being “detected.”

Together, these two concepts provide a logical resolution of the “least detectable amount” issue. Because of measurement error, some positive readings are inevitable, even at true zero concentrations, so \( L_C \) is a value for measured \( \hat{x} \), large enough that true zero-concentration specimens are very unlikely to produce such a large value for \( \hat{x} \) and be “detected.” It will then be true that specimens with very small values of \( \theta \) also have only small probabilities of detection. But large enough values of \( \theta \) lead to more frequent measured values of \( \hat{x} \) exceeding \( L_C \) (and therefore to detection). We have given the name \( L_Q \) to the least value of \( \theta \) that is “nearly sure” (i.e., has probability 1 − \( \beta \)) to produce a value of \( \hat{x} \) that results in detection.

The idea of “quantitative determination” is captured in a third and final concept.

Third Concept: Lower Limit of Quantitative Determination, \( L_Q \)

If the measurement error is of less and less relative size at higher concentrations \( \theta \), then one can seek to find a limit,
beyond which the relative error remains smaller than a prechosen fraction, Q. Currie (1) denotes this limit $L_Q$.

All three of these concepts are illustrated in the example below.

**Materials and Methods**

The RIA for digoxin, as performed routinely in the Drug assay Laboratory of our clinical laboratory, involves use of [122-3H]digoxin (New England Nuclear, Boston, MA), digoxin antibody prepared according to the method of Butler and Chen (3), dextran-coated charcoal separation of free and bound drug, and liquid scintillation counting of the radioactivity of the bound fraction with automatic external standard quench correction (4). Volumes of samples and standard are 0.5 mL; concentrations of the standards range from 0.3 to 5.0 µg/L, and the standard curve is determined by a least-squares linear regression of reciprocal counts vs concentration, for which we used a HP 9825A desk-top computer (Hewlett Packard Co., Palo Alto, CA). The digoxin-free "zero" samples described in the example were 0.5-mL aliquots of pooled blood-bank plasma, assayed one at a time.

**Example**

**Data:** To assess the sensitivity of the RIA for digoxin in our hands, we collected the following data. First, on five different days, we assayed, in duplicate, samples for standard curves (at 0.3, 1.0, 2.0, 3.0, and 5.0 µg/L) and two samples at 0 concentration. Thus, each of the five experiments had 12 data points. We took as the observed response $y = 10^6 \times (\text{dpm})^{-1}$, summarized in Table 1. As expected, the regression of $y$ on concentration is linear over the range 0.3 to 5.0 µg/L in each experiment.

Secondly, in each of 65 consecutive routine assays made during 30 days we included a single unidentified zero-concentration sample, the concentration of which, $x$, was estimated from the standard curve computed for the assay in which it was included. These 65 determinations (each of which would be zero if there were no measurement error) are summarized in Table 2.

**Results**

Graphs of the data in Table 1 (Figure 1) showed that the variability in $y$ was greater at the higher values of $x$. This changing variability in $y$ suggested the use of a weighted regression. We fitted each line separately, using weights based on the pattern of increasing variability estimated from all five experiments. In particular, the weights were chosen appropriately for

$$\sigma(y|x) = 1.4 + 4\theta$$

(1)

Using the weights given by expression 1, we fitted the five lines individually to the data in Table 1, as shown in Figure 1. The values of $\hat{a}$ (fitted value for $\theta = 0$) and $\hat{b}$ (the slope) are listed in Table 3, along with the observed values of $y$ for the two zero-dose samples. Ideally these values would equal (or be near to) the value of $\hat{a}$ for the same experiment. Instead, all 10 are larger, an indication that linear regression did not extend below the 0.3 µg/L sample all the way to zero.

The 65 values of $\hat{x}$ summarized in Table 2 had a mean of 0.021 µg/L and a standard deviation of 0.097 µg/L. If these data were from a normal distribution, one would estimate $L_C$ (the value exceeded with probability $a = 0.05$) as $0.021 + 1.65 \times 0.097 = 0.18$. Without assuming normal distribution, 0.20 is a good estimate of $L_C$, because the third highest value (among the 65) was 0.21 and the fourth highest was 0.18. Results of the two approaches agree well here. If there

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**Table 1. Results $[10^6 \times (\text{dpm})^{-1}]$ of Assay for Digoxin**

<table>
<thead>
<tr>
<th>Day</th>
<th>0</th>
<th>0.3</th>
<th>1.0</th>
<th>2.0</th>
<th>3.0</th>
<th>5.0</th>
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<tr>
<td>1</td>
<td>95.1</td>
<td>116.6</td>
<td>175.2</td>
<td>227.8</td>
<td>341.9</td>
<td>512.3</td>
</tr>
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<td>2</td>
<td>97.1</td>
<td>122.9</td>
<td>177.9</td>
<td>267.4</td>
<td>351.7</td>
<td>532.5</td>
</tr>
<tr>
<td>3</td>
<td>88.9</td>
<td>105.4</td>
<td>168.5</td>
<td>241.7</td>
<td>312.7</td>
<td>442.9</td>
</tr>
<tr>
<td>4</td>
<td>87.5</td>
<td>106.0</td>
<td>162.0</td>
<td>247.9</td>
<td>318.8</td>
<td>429.0</td>
</tr>
<tr>
<td>5</td>
<td>99.0</td>
<td>111.2</td>
<td>162.3</td>
<td>252.5</td>
<td>295.0</td>
<td>483.1</td>
</tr>
<tr>
<td>6</td>
<td>95.2</td>
<td>104.0</td>
<td>163.9</td>
<td>229.4</td>
<td>318.5</td>
<td>444.4</td>
</tr>
<tr>
<td>7</td>
<td>88.8</td>
<td>105.0</td>
<td>162.3</td>
<td>235.5</td>
<td>324.0</td>
<td>467.3</td>
</tr>
<tr>
<td>8</td>
<td>85.7</td>
<td>104.6</td>
<td>151.7</td>
<td>254.0</td>
<td>326.7</td>
<td>455.8</td>
</tr>
<tr>
<td>9</td>
<td>93.3</td>
<td>106.3</td>
<td>169.5</td>
<td>254.5</td>
<td>341.3</td>
<td>482.6</td>
</tr>
<tr>
<td>10</td>
<td>89.1</td>
<td>104.3</td>
<td>167.0</td>
<td>248.4</td>
<td>312.3</td>
<td>466.4</td>
</tr>
</tbody>
</table>

**Table 2. Estimated Concentrations ($\hat{x}$) for 65 Consecutive Zero-Concentration Samples**

<table>
<thead>
<tr>
<th>Value</th>
<th>Frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td>−0.15</td>
<td>3</td>
</tr>
<tr>
<td>−0.10</td>
<td>9</td>
</tr>
<tr>
<td>−0.05</td>
<td>8</td>
</tr>
<tr>
<td>0</td>
<td>13</td>
</tr>
<tr>
<td>0.05</td>
<td>15</td>
</tr>
<tr>
<td>+0.10</td>
<td>5</td>
</tr>
<tr>
<td>+0.15</td>
<td>8</td>
</tr>
<tr>
<td>+0.20</td>
<td>2</td>
</tr>
<tr>
<td>+0.25</td>
<td>2</td>
</tr>
</tbody>
</table>

Mean: 0.021  
SD: 0.097  
Minimum: −0.16  
Maximum: 0.26  

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Fig. 1. Results from five repetitions of a digoxin assay (Table 1), with their fitted lines (Table 3)
were strong evidence that $\hat{x}$ was normally distributed, we would prefer the estimate based on that information. In continuing with the example, we shall use the value 0.20.

Finding $L_Q$ requires determining that value of $\theta$ for which the $\hat{x}$ values produced by the assay for samples with concentration $\theta$ are very likely (we take $1 - \beta = 0.90$) to exceed the threshold $L_C$ for us, (0.20). The computation is, in fact, a balancing of the slope of the line with the value of $\sigma(y|\theta)$. We shall use in our calculation the line

$$y = 85 + 80\theta$$  

which is quite concordant with the five sample lines. We also shall use the earlier relation (equation 1) between $\sigma(y|\theta)$ and $\theta$.

The standard theory for bioassay with weighted least squares leads to the following estimated standard error for $\hat{x}$:

$$\sigma(\hat{x}) = 1/b \sqrt{\sigma^2(y|\hat{x}_0)}/m + \sigma^2(y)$$  

In this expression, we have written $m$ for the number of repeated samples of the unknown used in the assay; we have also treated the variability of $b$ as negligibly small, which is justified by the data of this example, since $\sigma(b)$ is 0.58—less than 1% of the value of $b$, which is 80. From the least-square analysis of the five lines (Table 3), the value 5.0 emerged as the estimate of $\sigma^2(y)$.

The value of $L_Q$ is determined as that value of $\theta$ for which the following equation holds:

$$\theta = L_C + 1.28(1/b)\sqrt{\sigma^2(y|\theta)/m + \sigma^2(y)}$$  

In expression 4, the number 1.28 is the upper 10th percentile of the standard normal distribution, reflecting our choice of $\beta$ as 0.10, and using the assumption that the assay values $y$ are normally distributed. For this example, $L_C$ is 0.20, $\sigma^2(y) = 5.0$, $b = 80$, and $\sigma(y|\theta) = 1.4 + 4\theta$; we then find $L_Q$ as the solution $\theta$ of the equation:

$$\theta = 0.20 + (1.28/80)\sqrt{(1/m)(1.4 + 4\theta)^2 + 5}$$  

The solution for $m = 1$ is 0.253, and for $m = 2$ is 0.245.

We interpret these results as follows: First, if a single specimen has an actual concentration of $\theta = 0.253$, we have a 90% chance of obtaining an estimated value $\hat{x}$ that exceeds 0.20; thus, we have a 90% chance of "detecting" a specimen having a true concentration of 0.253 or more. Second, if duplicates of unknowns are used, then this 90% chance applies to a specimen with a true concentration of only 0.245 or more.

Finally, we estimated $L_Q$, the value of $\theta$ for which $\sigma(\hat{x}) = 0.10$. This value has the following interpretation: For specimens with concentrations exceeding $L_Q$, the standard error

of the resulting assay measurement is at most 10% of the actual concentration. The value of $L_Q$ is therefore that $\theta$ for which

$$\sigma(\hat{x}) (1/\theta) = 0.10$$  

Using expression 3, we may write this as

$$\frac{1}{b}\sqrt{\frac{1}{m}\sigma^2(y|\hat{x}_0)} + \sigma^2(y) = \theta 10$$  

and inserting, as before, values for $\beta$, $\sigma^2(y)$, and $\sigma^2(y|\hat{x}_0)$, we have $L_Q$ as the solution $\theta$ of

$$\frac{1}{80}\sqrt{1/m(1.4 + 4\theta)^2 + 5} = \theta 10$$  

The solution of this equation is $L_Q = 0.46$, if $m$ is 1, and 0.35 for duplicates ($m = 2$).

If we desired to define $L_Q$ so that two standard errors of $\hat{x}$ were equal to 0.16, then for $m = 1$ no such value for $L_Q$ exists, because the left-hand side of equation 8 always exceeds $\theta 20$ for $m = 1$. For $m = 2$, a solution is possible; it is 1.28.

**Discussion**

The methods illustrated in this paper depend upon three conditions:

- The regression of reciprocal counts on concentration is linear, with normally distributed errors.
- The standard deviation of response is a known function of concentration (or one that can be well estimated), so that weighted least squares may be applied.
- The assay produces a very tightly determined line, permitting variability in the estimated slope to be neglected.

The same assay performed in other laboratories probably would yield different (though perhaps not greatly different) values for $L_D$, $L_C$, and $L_Q$. The determination of assay sensitivity is—and should be—a separate undertaking for each laboratory.

We would have hoped that the relation $\sigma(y|\theta) = 1.4 + 4\theta$, found in our model experiments to hold for $\theta = 0.3$, 1.0, 2.0, 3.0, and 5.0, would also have held at 0, but it did not. If it had held, we should have found $\sigma(y|0) = 1/80\sqrt{(5.0 + (1.4)^2} = 0.033$. Instead, we found for 65 values the much larger value $\sigma(y|0) = 0.097$. We regard this discrepancy as a challenge to us to search out the causes of this greater variation at zero.

Interlaboratory comparison based on standard designs and methods of analysis can help in identifying, tracking down, and enabling correction of inadequacies in assay precision and accuracy. The net outcome, then, is improved service by clinical chemistry laboratories.

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**References**