Dilution as a Source of Error: Implications for Preparation and Calibration of Laboratory Standards and for Quality Control of Radioimmunoassays

Rose E. Gaines Das

Although dilution of materials is generally accepted to involve some error, workers often assume that this error may be discounted. However, comparing solutions of two identical materials diluted in the same way shows that dilution errors can cause a considerable departure from the expected identity. I describe this effect and its dependence on the errors at various stages of the dilution process.

Additional Keyphrases: statistics - variation, source of - analytical error

When materials, especially standards, are prepared for use in systems such as radioimmunoassays, a major consideration is the need to dilute the standard so that the solution for assay contains the analyte in a concentration similar to that observed in clinical samples. Any measurement such as pipetting involves some error, but the magnitude of the error is likely to be underestimated. Moreover, even though the coefficients of variation associated with each step of a dilution process may be small, their cumulative effect on the concentration of material at the final dilution can be a dramatic increase in variability. For precise assays the variability due to dilution errors may considerably exceed that due to the analytical error, i.e., the measurement error inherent in the assay method. Thus use of the analytical error alone to determine the precision of potency estimates can be misleading.

In this paper I discuss the cumulative effect of small errors in sequential dilution procedures on the coefficient of variation of the final estimate of relative potency of two solutions of the same material prepared in the same or a similar way.

Theory

Standard Materials

International as well as many national biological standards and reference materials are stored in ampoules, each of which is stated to contain a specified amount of the material (1). For use in assays, the contents of the ampoule of standard material are dissolved in a measured volume of diluent, the resulting solution is then subdivided into measured portions, and each portion is subsequently further diluted and then used in an assay. (See, for example, reference 2.) When the material from the ampoule has been used up, the contents of another ampoule are similarly reconstituted and used. Thus, the coefficient of variation of the estimate of potency for portions of two ampoules of such a reference material compared in an assay is of practical importance, particularly when the reference materials are used as laboratory standards and the comparison is for the purpose of calibrating a replacement laboratory standard. If the replacement standard is not correctly calibrated, discontinuity of values estimated by comparison with it can result, and comparisons of estimates made with use of old and replacement laboratory standards can be invalidated.

Variance of Potency Estimates

The relative potency, r, of two identical ampoules of the same material diluted in the same way should be 1. If R is an estimate of r obtained from an assay, usual methods of estimation assume that log R is normally distributed with mean log r = 0 and standard deviation Cq. For radioimmunoassays Cq, approximately the coefficient of variation of R, is of the order 0.02. However, each dilution step involves error, and r will not be precisely 1 but will itself be subject to variation, depending on the dilution errors. The variance of log R will thus be increased by S2, the variance of log r (see Appendix).

Clearly, estimates of variation of R based only on the analytical error for the assay method, Cq2, are underestimates. Table 1 lists values of S, the standard deviation of the log relative potency resulting only from dilution errors. These have been calculated by assuming, for simplicity, that the same coefficients of variation apply for each volume measured and the same dilution factors are involved at each step and for each ampoule of material. That is, log R would have standard deviation S even if there were no analytical error in the assay method. Thus, coefficients of variation for R of 10% or greater are not surprising, even though the analytical error may only suggest a coefficient of variation of 2%. From inspection of the Table it is clear that making few dilution steps, each with a large dilution factor, and taking care in the measurement of...
Table 1. Values of S, the Standard Deviation of the Log Potency (Deviations Resulting from Dilution Errors)\(^a\)

<table>
<thead>
<tr>
<th>No. of dilution steps</th>
<th>1</th>
<th>1.5</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>Dilution of initial soln obtained</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1000-fold dilution</td>
<td>100-fold dilution</td>
<td>100- to 10-fold dilution</td>
<td>10-fold dilution</td>
<td>2-fold dilution</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>0.0264</td>
<td>0.0529</td>
<td>0.0793</td>
<td>0.1058</td>
<td>1:1000</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>0.0263</td>
<td>0.0526</td>
<td>0.0789</td>
<td>0.1052</td>
<td>1:100</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>0.0329</td>
<td>0.0659</td>
<td>0.0988</td>
<td>0.1317</td>
<td>1:10 000</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>0.0384</td>
<td>0.0768</td>
<td>0.1153</td>
<td>0.1537</td>
<td>1:1 000 000</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>0.0319</td>
<td>0.0638</td>
<td>0.0956</td>
<td>0.1275</td>
<td>1:1000</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>0.0250</td>
<td>0.0500</td>
<td>0.0749</td>
<td>0.0999</td>
<td>1:10</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>0.0308</td>
<td>0.0616</td>
<td>0.0924</td>
<td>0.1232</td>
<td>1:100</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>0.0357</td>
<td>0.0713</td>
<td>0.1070</td>
<td>0.1427</td>
<td>1:1000</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>0.0399</td>
<td>0.0799</td>
<td>0.1198</td>
<td>0.1598</td>
<td>1:10 0000</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>0.0438</td>
<td>0.0876</td>
<td>0.1315</td>
<td>0.1753</td>
<td>1:100 000</td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>0.0474</td>
<td>0.0947</td>
<td>0.1421</td>
<td>0.1895</td>
<td>1:1 000 0000</td>
<td></td>
</tr>
</tbody>
</table>

\(^a\) A common coefficient of variation, C, is assumed for each volume measured, and a common dilution, d, is assumed for each of k dilution steps taken to achieve each of the two solutions compared.

\(^b\) Two steps with dilution factors of 100-fold or 10-fold, in either order.

volumes (thus reducing coefficients of variation) will give the smallest values of S. This can be illustrated by comparison of the values of S shown for different ways of obtaining a 1000-fold dilution. Of these, a single step with this dilution factor has the smallest coefficient of variation. However, practical considerations—for example, the difficulty in achieving a homogeneous mixture with two such different volumes—must also be taken into account.

Table 1 also illustrates the consistent increase in variability with each step in a serial dilution process. For example, after 13 steps of a twofold dilution process the standard deviation for the resulting solution has doubled. If, of two solutions being assayed, one has required greater dilution than the other, then the solution requiring the greater dilution may have a more variable estimate of potency.

**Discussion**

The variability of potency estimates of the same material obtained from different assays in the same laboratory and from different laboratories forms the basis of many criteria for determination of “quality” for internal and external quality-control schemes. Nevertheless, the role of the laboratory standard or reference material used in radioimmunoassays and the requirement that the standard should be as nearly constant as possible over a series of assays have been neglected in many investigations into sources of variability of estimates, perhaps because it has been assumed that the standard is constant and requires no further attention. Siddiqui and Craig (3), for example, devote only a short paragraph to “the standard,” none of which is concerned with its preparation for individual assays. New batches of laboratory standard will, however, periodically have to be prepared in any laboratory carrying out radioimmunoassays; the potential for discontinuity when two identical preparations of human prolactin were compared has been discussed elsewhere (2). Booth et al. (4) concluded that the variance of laboratory standards was equal to or greater than that of quality-control results; discontinuity resulting from dilution procedures is likely to contribute to this variance. Nevertheless, the discontinuity in estimates that can result when a laboratory standard is replaced with a fresh solution of essentially identical material is not sufficiently widely appreciated. If the standard material must be weighed or otherwise prepared, additional variability is likely to be introduced. Moreover, the magnitude of the error introduced by imprecise pipetting during the preparation of the standard, particularly when several successive steps are involved, is often underestimated, and there may be considerable surprise when large discon-
tinuities result. Siddiqui and Craig (3) state that, “Repeated pipetting of each reagent should show reproducibility in the order of 1%,” although laboratory evaluation of several pipettes in their study showed coefficients of variation of 2%. Midgley et al. (5) report that choice of micropipetting equipment can make a threefold difference in error, and also report that coefficients of variation associated with several pipettes are about 2%, ranging from less than 1% to nearly 5% depending on the technician and pipette. The nature (e.g., the viscosity) of the solution pipetted may also affect the coefficient of variation. When allowance is made for additional assay errors due to such factors as constraints of time and incomplete mixing of materials, the reported coefficients of variation almost certainly underestimate the coefficients of variation obtained in practice.

An example of the possible effect of dilution errors is shown in the report of an international collaborative study of the International Reference Preparation of human prolactin for immunoassay (2). In that study participants compared estimates of prolactin in two identical ampoules coded variously as X and IRP. In each of 23 assays R, the relative potency of X in terms of the IRP, was computed (Figure 1). The observed mean of log R is 0.02, compared with E (log R) = 0 (Appendix); with the expected mean of 0, the observed variance of log R is 0.0079 (or 0.0078 with the observed mean). The average estimated analytical error for the 14 participants was 0.0004 (i.e., CR = 0.02). Thus these data suggest that S is approximately 0.086. Figure 1 also shows that the normal curve obtained from the expected mean log relative potency of 0 and the observed standard deviation, 0.088, fits the data reasonably well, whereas the normal curve obtained from the expected mean of 0 and the observed analytical error of 0.02 would predict a less-scattered distribution of potency estimates than was observed. The results of a collaborative study of ampoules of a human insulin C peptide analog (6) also showed a large value of S (approximately 0.039) when compared with a CR of approximately 0.01.

The present analysis indicates that for the individual labor-

atory, between-assay variation may be decreased by using working standards that require the fewest dilution steps—by preparing a large homogeneous solution of reference material; by dividing the solution into portions precisely measured to provide the quantity required for individual assays; by storing the portions under optimum conditions for stability; and by calibrating several of the portions carefully before use. The possibility of discontinuity when a replacement standard is prepared must not be ignored, nor can within-laboratory between-assay variation be considered representative of between-laboratory variation when different laboratories use different solutions of the same standard or reference material.

I thank Miss M. V. Mussett, Dr. P. Mary Cotes, and Dr. D. R. Bangham for helpful comments.

Appendix

Derivation of the Variance of Potency Estimates in the Presence of Dilution Errors

Assume that an ampoule contains precisely U units of the standard material, which are dissolved in an initial volume V1 of diluent to give a solution with concentration U/V1. This solution is then subdivided into portions, each of volume a, which are each diluted with volume v of diluent to give a solution for an assay with concentration U/v where d denotes the dilution factor a/(a + v). If more than one dilution step were necessary, then for the ith step, portions of volume ai would be taken from the solution resulting from the previous step, and diluted with volume vi of diluent. The dilution factor for the ith step would be di = ai/(ai + vi) and the concentration of the final solution after k dilution steps would be diU. If log Y is a normally distributed random variable with variance C2, then for small values of C, the coefficient of variation of Y is approximately C. The error in dilution procedures can thus be expressed in terms of random variables Vi, V, and A, corresponding to the desired values vi, v, and a (each multiplied by a normal random variable with a mean of 1) and having coefficients of variation Ci, Cv, and CA (the standard deviations of the multiplicative random variables), respectively, and random variable D = A/(A + V), corresponding to the dilution factor d. In the more general case of k dilution steps in series there would be, corresponding to each step, variables Vi, Ai, and D = Ai/(Ai + Vi). Chase and Hoel (Biometrika 62: 329–344, 1975) have shown that for the range of d, Cv, and CA of interest here (namely, for d in the range of 0.001 to 0.5 and Cv and CA in the range of 0.01 to 0.05), log D is distributed approximately as a normal variable with mean log d and variance (1 - d)2(Cv2 + Ca2). Similarly, log V1 is distributed approximately normally with mean log v1 - C2/2 and variance 3C2/2.

If the concentration of the standard solution is defined as X = DU/V1, then the mean of log X is

\[ \log U + \log d - \log v1 + C1^2/2 \]

and the variance of log X is

\[ (1 - d)^2(Cv^2 + Ca^2) + 3C^2/2. \]

For the more general case of k dilution steps, \[ X = D1 \ldots DkU/V1 \]

and the variance of log X is

\[ \sum_i (1 - d_i)^2(Cv_i^2 + Ca_i^2) + 3C^2/2. \]

If Xp and Xr represent the concentrations of solutions of
the contents of two ampoules, P and N, respectively, then the relative potency is

\[ r = \frac{X_P}{X_N}. \]

If each solution has been prepared from ampoules of identical material with the same dilution steps, then \( \log r \) is approximately normally distributed with a mean of 0 and variance

\[ S^2 = 2(1 - d)^2(C_Y - C_X)^2 + 3C_Y^2, \]

or in the more general case of \( k \) dilution steps,

\[ S^2 = 2 \sum_{i=1}^{k} (1 - d_i)^2(C_Y^i - C_X^i)^2 + 3C_Y^2. \]

A similar expression would result if different dilution steps were used for each ampoule or different coefficients of variation were used for different dilution steps or solutions. The sum above \( (S^2) \) would include terms \((1 - d)^2(C_Y^2 + C_X^2)\) with appropriate values of \( d, C_Y \) and \( C_X \) for each step.

If \( R \) is the estimate of the relative potency computed for an assay, then for known relative potency, \( r \), and usual methods of estimation, \( \log R \) is normally distributed with respective mean and variance of

\[ E(\log R | r) = \log r \]

and

\[ \text{Var}(\log R | r) = C_R^2. \]

However, if \( r \) is not known but, because of dilution errors occurring in the preparation of the two solutions, may vary as described above, then \( \log R \) will be normally distributed with mean and variance

\[ E(\log R) = 0 \]

and

\[ \text{Var}(\log R) = S^2 + C_R^2. \]

References


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**Effect of Detergent on Kinetic Jaffé-Method Assay of Creatinine**

**Anders Bergman and Gösta Öhman**

Using isotope dilution–mass spectrometry as a reference method, we show that the kinetic Jaffé method for determination of serum creatinine sometimes produces results that are much too high. Such falsely increased values were obtained in about 10% of the sera collected for routine determination of creatinine, and were most common when certain types of collecting tubes were used. The interferences could be overcome by use of detergent in the reaction mixture.

The specificity of the Jaffé reaction for the determination of serum creatinine can be improved by use of a kinetic evaluation of the color development. The rate of reaction of alkaline picrate with different interferring endogenous compounds has been studied carefully. In the early phase, different fast-reacting interferences contribute to the color development. About half a minute after the start, the reaction seems to be almost exclusively due to creatinine itself. In the late phase of the reaction, the color development is influenced by slow-reacting interferences (1–7).

In our laboratory we use a modification of the Jaffé reaction that measures the color development during the interval 25 to 35 s after the start of the reaction. Sometimes this method gives erroneously high results, as judged by comparison with results from a highly specific mass-fragmentographic reference method (8). These increases were most frequently observed in sera collected from kidney-transplant patients. Also in other patient populations, however, sudden false increases sometimes occurred. A similar increase was also found sometimes when some specific types of material were used in the separation of serum from erythrocytes during centrifugation; such interference only occurred with some sera. In general, the interference was not stable, and after storage of the sample for some days at 4 °C most of it disappears.

Heinşegård et al. (9) showed that deproteinization can be avoided by the addition of detergent, when measuring creatinine with the end-point Jaffé method. Surprisingly, we found that the use of detergent in the reaction mixture also completely eliminates the type of interference referred to above.

**Materials and Methods**

Blood was collected in tubes purchased from Becton-Dickinson, Rutherford, NJ 07070. Two types of tubes were used. The Vacutainer SST (cat. no. 03810) contains an "inert