Error Estimation in the Quantification of Alkaline Phosphatase Isoenzymes by Selective Inhibition Methods

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A method for calculating, by selective inhibition, the activities in serum of isoenzymes of alkaline phosphatase (EC 3.1.3.1) originating from bone, liver, intestine, and placenta produced results for a sample of patients for which the imprecision was five- to 25-fold higher than that of the alkaline phosphatase method used—too imprecise for routine clinical use. Error analysis by direct calculation and by Monte Carlo estimation revealed that the algorithm used in the method completely accounted for the increase in imprecision of the isoenzyme estimations. I recommend that all methods involving such algorithms or the principle of multicomponent analysis should undergo a thorough error analysis by use of the techniques described here, to obtain an estimate of the increase in imprecision that is ascribable to the particular numerical technique used.

Alkaline phosphatase (EC 3.1.3.1) isoenzymes are inhibited to various extents by treatment with (e.g.) L-phenylalanine, urea, L-tryptophan, L-homoarginine, levamisole, brometramisole, guanidine hydrochloride, EDTA, heat, and low pH (1-4). Heat inactivation at 65 °C is the only completely specific way to determine the activity remaining after such treatment, the other sources of activity being completely destroyed. All other inhibitors show various degrees of inhibition for each isoenzyme, and none is completely specific for a single isoenzyme.

Quantitative estimates of the isoenzymes are possible, however, by using the technique of multicomponent analysis, a technique used in spectrophotometry to resolve mixtures of components with overlapping but dissimilar spectra (5). The simple principle involved is that, if n components in a mixture have different absorbances at each of n different wavelengths, the contribution of each component may be found by the solution of n simultaneous equations. This can be applied to resolving the different amounts of alkaline phosphatase isoenzymes in a mixture if the n isoenzymes have different degrees of inactivation with n different inhibitors. Solution of the equations for a particular system will result in an algorithm with which the amount of each isoenzyme may be calculated from the activity of the mixture in the presence of each inhibitor. Such an algorithm has recently been described (6) for calculating the amount of placental, intestinal, liver, and bone isoenzymes in serum from the activities of the sample in the presence of L-phenylalanine, guanidine hydrochloride, and heat (65 °C for 10 min).

Using this method on patients’ samples, I found the scatter of results to be extraordinarily high for some isoenzymes, and the imprecision of the results to be unacceptably high for a routine method used to monitor cancer patients. This prompted an investigation into the errors generated by the algorithm and an attempt to estimate the minimum achievable precision for such methods.

Materials and Methods

Isoonzyme Determinations

Alkaline phosphatase isoenzyme assays were carried out in a Cobas Bio centrifugal analyzer according to the method of Shephard et al. (6), with AMP buffer ("Monotest Alkaline Phosphatase"; Boehringer Mannheim, Lewes, U.K.; cat. no. 396494).

Error Analysis

Direct calculation: The set of simultaneous equations used by Shephard et al. (6) to derive the algorithm was:

\[ T = P + I + L + B \]  
\[ H = 1.00P \]  
\[ G = 1.24P + 0.90I + 0.47L + 0.14B \]  
\[ A = 0.36P + 0.33I + 0.86L + 0.86B \]

By substituting for P, I, L, and B the placental, intestinal, liver, and bone isoenzymes, respectively; T is the total activity (no inhibitors); H is the heat-stable activity (after 65 °C for 10 min); A is the activity in the presence of phenylalanine (10 mmol/L); and G is the activity in the presence of guanidine hydrochloride (0.3 mol/L).

The algorithm used to calculate isoenzyme activities was:

\[ P = H \]  
\[ I = 1.6626T - 1.8868A - 0.9434P \]  
\[ L = -0.4242T - 2.3030I - 3.3333P + 3.0303G \]  
\[ B = T - (P + I + L) \]

By substituting for P, I, and L in the right-hand terms and combining terms, one may express each isoenzyme as a linear function of H, T, A, and G:

\[ P = H \]  
\[ T = 1.6626T - 0.9434H - 1.8868A \]  
\[ L = -4.1610T - 1.1607H + 4.3453A + 3.0303G \]  
\[ B = 3.5394T + 1.1041H - 2.4568A - 3.0303G \]

Assuming the errors in each measurement (T, H, A, G) are normally distributed and uncorrelated, the exact error variance (Var) for each isoenzyme determination may be calculated by the formula:

\[ \text{Var}(L) = \Sigma(a_i^2 \text{Var} x_i) \]

where \( L = a_0 + a_1 x_1 + a_2 x_2 + \ldots + a_n x_n \) for observations \( x_1, x_2, \ldots, x_n \) and the \( a_i \) values are constants (7). This gives the following error function for each isoenzyme:

\[ \text{VarP} = \text{VarH} \]
\[ \text{VarI} = 2.7642\text{VarT} + 0.8900\text{VarH} + 3.5600\text{VarA} \]
\[ \text{VarL} = 17.3139\text{VarT} + 1.3472\text{VarH} + 18.8816\text{VarA} + 9.1827\text{VarG} \]
\[ \text{VarB} = 12.5203\text{VarT} + 1.2190\text{VarH} + 6.0447\text{VarA} + 9.1827\text{VarG} \]
which relates the error in each measurement of alkaline phosphatase activity (T, H, A, G) to that in the calculated isoenzyme value. Standard deviation (SD) and coefficient of variation (CV) were derived from the variance estimate by standard formula $SD = \sqrt{Var}; CV (%) = (mean/SD) \times 100$.

Monte Carlo estimation: As a check on the derived variance equations, a computer was used to obtain a Monte Carlo estimate of the error distribution of each isoenzyme. Values of T, H, A, and G that would give appropriate or "true" mean values for P, I, L, and B were calculated from the original equations. For each trial, a random normally (gaussian) distributed error of known CV was added to the "true" value of T, H, A, and G and the corresponding values of P, I, L, and B were calculated by the algorithm. Each trial was conducted 999 times and the resulting error distributions for P, I, L, and B were calculated and plotted. These distributions constitute a Monte Carlo estimate of the true error distributions of P, I, L, and B, given the original error in T, H, A, and G. Normally distributed errors were obtained by multiplying the (known) SD for each value of T, H, A, and G by a standard normal variable, generated using the "polar method" of Box et al., as described by Knuth (8). The algorithm used to generate standard, normal distributed variables is given in the Appendix. I carried out the simulation on a British Broadcasting Corporation Master series microcomputer with a 6502A second processor.

Simulated Cases

To illustrate typical clinical situations where quantitative isoenzyme values would be used, I estimated imprecisions for theoretical patients with the following "true" isoenzyme values (U/L):

<table>
<thead>
<tr>
<th>Specimen</th>
<th>P</th>
<th>I</th>
<th>L</th>
<th>B</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0</td>
<td>10</td>
<td>50</td>
<td>50</td>
</tr>
<tr>
<td>2</td>
<td>0</td>
<td>10</td>
<td>50</td>
<td>100</td>
</tr>
<tr>
<td>3</td>
<td>0</td>
<td>10</td>
<td>50</td>
<td>200</td>
</tr>
</tbody>
</table>

Specimen 1 shows values in the normal range for all the isoenzymes and specimens 2 and 3 show successive doublings of the B isoenzyme. This could occur, for example, as a sequence in a patient who has a treated malignancy relapsing in bone over time, and in whom accurate and precise isoenzyme determinations may be taken as evidence of the occurrence and site of secondary tumors.

Statistics

The chi-squared statistic for the comparison of a variance, $Var_{obs}$, of sample size n, with a theoretically expected variance, $Var_{exp}$, is $(n - 1)Var_{exp}/Var_{obs}$ with $n - 1$ degrees of freedom (df). The F-statistic for the comparison of two variances $Var_1$, $Var_2$ is $Var_1/Var_2$, where $Var_1$ is the larger of the two variances, with $n_1$ and $n_2$ df (9).

Results

Modelled Findings

Comparison of observed and expected imprecisions: The imprecisions obtained in 25 repeated determinations of P, I, L, and B isoenzymes for a single patient's specimen are shown in Table 1. The CVs of the isoenzyme estimates are five- to 25-fold higher (9.25–42.86%) than the CVs of the initial determinations of T, H, A, and G (i.e., 1.46–2.32%). This result was typical and resulted in many determinations that produced "impossible" results with specimens (i.e., isoenzyme activities greater than the total activity, or with "negative" activity). Using the means and variances obtained for T, H, A, and G, I calculated the theoretically expected variances directly or by Monte Carlo estimation. The experimental results did not differ significantly from either of these estimates (Table 1).

Achievable imprecision of isoenzyme assays: The relationship between the imprecision of the initial measurements T, H, A, and G ("initial assay CV") and the imprecision of the calculated isoenzyme activities ("isoenzyme CV") was then determined for each case by using the variance equations, equations 14–17, and by Monte Carlo estimation. The results were almost identical; the Monte Carlo estimates for specimens 1 and 3 are shown in Figure 1. The isoenzyme CV is a linear function of the initial assay CV, but the slope of the relationship differs in each case, depending on the particular mixture of isoenzymes. The graphs give an estimate of the imprecision a laboratory may expect from these isoenzyme assays, in these particular cases, given its alkaline phosphatase assay imprecision. If we take as an "average laboratory" imprecision a CV of 3.4% [the median CV in a recent international quality-assessment scheme (12)], the CV of the B isoenzyme falls from 31% to 19%, the L isoenzyme increases from 42% to 100%, and the I isoenzyme increases from 82% to 208% in samples 1 and 3, respectively, where the only change in the isoenzyme mixture is an increase of the B isoenzyme from 50 to 200 U/L. An estimate of the precision required in the initial alkaline phosphatase assay to achieve any required precision of isoenzyme determination can also be made from these graphs. If we apply to the isoenzyme assays a proposed analytical goal of 3.4% for alkaline phosphatase assays (12), a minimum initial CV of 0.5% would be required to achieve this for all three isoenzymes in specimens 1, 2, and 3. Replication of assays will reduce imprecision by a factor of $1/\sqrt{n}$, where n is the number of replicates. However, a minimum of 46 replicates would be required to achieve this analytical goal if our assay CV was 3.4%.

Effect on Clinical Interpretation

Monitoring change: The effect of these huge increases in the isoenzyme imprecisions is best shown by considering the 95% confidence interval (CI) for the difference between two results on the same specimen (2.77 SD). If we use the "average laboratory" initial CV of 3.4% and the 95% CI as a criterion of a significant change, for the B isoenzyme in the normal range (specimen 1) a change of 44 U/L would be significant, and in the abnormal sample, specimen 3, a
Fig. 1. Relationship between imprecision of alkaline phosphatase assay (initial assay CV) and isoenzyme CV derived from the variance equations for the algorithm described in the text for I (---), L (----), and B (-----) isoenzymes.

The 3.4% CV values, representing the analytical goal and average laboratory CV, are marked on the y- and x-axes, respectively. A, specimen 1; B, specimen 3.

change of 102 U/L. However, the corresponding figures for the total alkaline phosphatase assay would be 10 U/L (specimen 1) and 25 U/L (specimen 3). The direct total assay would be more sensitive.

Detecting the predominant isoenzyme: Identifying the source of the predominant isoenzyme is illustrated in Figure 2, which shows the frequency distribution of results obtained for each isoenzyme in specimen 2 for various initial CVs (1%, 3.4%, and 5%). Specimen 2 is the most appropriate for this illustration because the B isoenzyme alone has doubled and the resulting increase in total alkaline phosphatase activity would prompt an investigation into the tissue source of the enzyme. Taking the upper limit of the reference range for the L, B, and I isoenzymes as 74, 60, and 15 U/L, respectively (6), we see that, at a 3.4% initial CV, only 42% of results would show the B isoenzyme alone above the reference range and 4% would show the L isoenzyme alone. All three isoenzymes would be clearly separated only at an initial CV <1%.

Comparison of imprecisions obtained by using the algorithm and a numerical technique: Equations 1–4 reduce to three simultaneous equations in three unknowns (by eliminating equation 2), which may be solved by using one of a number of numerical methods. Table 2 compares the results of a Monte Carlo estimation of the imprecision obtained for B, L, and I isoenzymes in specimen 2 by using the algorithm and the gaussian elimination technique (10). Again, the initial assay CV was 3.4%. There was no significant difference between imprecisions obtained by either technique (Table 2).

Discussion

Algorithms derived from multi-component inhibition systems have been used before for isoenzyme determination. Determination of alkaline phosphatase isoenzymes B, L, and I by phenylalanine and urea inhibition in a three-component system has been described (13–15). Bergstrom and Lefvert (16) noted for this system that the range of results obtained for a given initial assay CV of 4.5% was very wide, but made no thorough error analysis. A more recent example for another enzyme is the two-component system for alpha-amylase isoenzymes, with use of wheat-germ protein inhibition (17). Instead of deriving algorithms for these systems, the original set of simultaneous equations could be solved by using gaussian elimination, Gauss-Jordan, or matrix inversion techniques on a computer. The error analysis may be quite complex, but suitable error estimates can still easily be found by using the Monte Carlo method described here without any resort to complex mathematical analysis, as shown above.

The method described here is a more recent application of the technique, which still suffers from the problem of an undefined increase in imprecision of the derived isoenzyme results. Imprecision values obtained by repeated estimation of one or two isoenzyme mixtures are quoted (6, 13–15) but, as is quite clear from the results here and in reference 16, in practice, they can be much higher. An error analysis defines exactly the relationship between initial assay imprecision and isoenzyme imprecision. As shown here by direct calculation and Monte Carlo estimation, the increase in imprecision can be completely accounted for by propagation of errors in the algorithm or numerical technique used. Error analysis of this method (6) not only shows that it is unlikely in most laboratories to produce acceptably precise results for monitoring patients or for clearly indicating the probable source of an increase in alkaline phosphatase activity in clinically relevant situations, but also defines the degree of precision necessary to achieve those ends. The final error

Table 2. Comparison of imprecisions (U/L) Obtained for Specimen 2 by Monte Carlo Estimation by Using the Algorithm and Gaussian Elimination

<table>
<thead>
<tr>
<th>Algorithm</th>
<th>Variance</th>
<th>SD</th>
<th>CV, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>B</td>
<td>152.22</td>
<td>12.34</td>
<td>116.70</td>
</tr>
<tr>
<td>L</td>
<td>934.33</td>
<td>30.07</td>
<td>63.06</td>
</tr>
<tr>
<td>I</td>
<td>116.70</td>
<td>12.34</td>
<td>116.70</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Gaussian elimination</th>
<th>Variance</th>
<th>SD</th>
<th>CV, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>B</td>
<td>156.89</td>
<td>12.53</td>
<td>116.70</td>
</tr>
<tr>
<td>L</td>
<td>875.63</td>
<td>29.59</td>
<td>63.06</td>
</tr>
<tr>
<td>I</td>
<td>473.51</td>
<td>21.76</td>
<td>116.70</td>
</tr>
</tbody>
</table>

Initial assay CV = 3.4%. F-ratios for each isoenzyme were intestinal 1.031, liver 1.067, bone 1.126—not significantly different at P = 0.05 for a two-tailed test and 998 df for each variance.
function (equations 14 to 17) consists of terms that represent the errors in the original measurements (VarT, VarH, etc.) and coefficients that are determined by the inhibition coefficients (% inactivation) for each isoenzyme and inhibitor. It should be possible, therefore, to select or design a set of inhibitors that would give acceptably precise results.

Indeed, this is shown in a later modification (18) of the method discussed above, with use of levamisole (10 mmol/L) instead of L-phenylalanine. This considerably reduces the imprecision of determinations of the I isoenzyme. Replacing equation 4 above by one for determining levamisole inhibition in this system—\( A = 0.39P + 0.62I + 0.013L + 0.013B \), where \( A \) is now the levamisole-inhibited activity—or substituting for equation 6 with the new algorithm given by Peake et al. (18), and carrying out the error analysis described above will precisely define the decrease in imprecision. For specimen 3, the CV of the I, L, and B isoenzymes is reduced by 97\%, 87\%, and 62\%, respectively.

This error analysis has been concerned mainly with the propagation of imprecision and is not really complete. The inhibition coefficients have been assumed to be exact here, and the inhibitors also have been assumed to react under the conditions of the assay in the same way as when they were used to determine the inhibition coefficients. Neither of these assumptions is necessarily true, and errors in the coefficients can significantly affect the accuracy of the results, depending on the particular system of equations (19). It is probably wise to check the values yourself before applying an algorithm or a numerical technique.

In summary, multicomponent analysis should be used with some caution when applied to measurements of isoenzymes by multiple inhibition methods, especially when the measured values used are subject to significant error. A suitable error analysis should be carried out for any particular algorithm or numerical technique used in such methods. This will give a clear estimate of the minimum achievable precision inherent in the method, against which the value of empirically obtained imprecisions may be judged.

Appendix

The algorithm given by Knuth (8) to obtain independent, normally distributed variables with mean = 0 and Var = 1 ("standard normal variables") is:

1. Generate two independent random variables U1 and U2, uniformly distributed between 0 and 1 (the RND function in most versions of BASIC will do this).
2. Set \( V1 = 2U1 - 1 \) and \( V2 = 2U2 - 1 \)
3. Set \( S = V1^2 + V2^2 \)
4. If S \( \geq 1 \), return to step 1
5. Calculate \( X1 = V1 \sqrt{-2 \ln S} \) and \( X2 = V2 \sqrt{-2 \ln S} \)

X1 and X2 are each the required standard normal variables.

References