Instrumental and Procedural Sources of Error in Determination of Bile Pigments in Amniotic Fluid

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Although the Liley spectrophotometric estimation of bile pigments in amniotic fluid [Amer. J. Obstet. Gynecol. 82, 1359 (1961)] is widely used, the levels of accuracy and precision necessary for clinically meaningful results have not been clarified. This paper delineates some of the important instrumental and procedural sources of error, and how each affects the final value of \( \Delta A_{445} \): (a) A limit of error propagation is presented to show that the uncertainty in \( \Delta A_{445} \) due to instrumental factors is considerably larger than is commonly appreciated. (b) It is desirable to use a logarithmic baseline estimation, as did Liley, instead of a linear estimation, which can introduce a serious bias. (c) Failure to establish a true zero-absorbance baseline before scanning the specimen can also result in a significant bias in the calculated \( \Delta A_{445} \).

Additional Keyphrases erythroblastosis fetalis • spectrophotometry • propagation-of-error formulas • diagnostic aid

Bile pigments in amniotic fluid, long determined in clinical laboratories, provide an index to how severely the fetus is affected in erythroblastosis fetalis. Despite the widespread use of this test, there is little emphasis in the literature on the levels of accuracy and precision required for clinically meaningful results.

This paper discusses some important instrumental and procedural sources of error that should be recognized in a method derived from that of Liley (1, 2). Briefly, the procedure involves measuring the absorbance of filtered amniotic fluid at 350, 455, and 550 nm. These wavelengths differ slightly from those given in Liley's original method. Instead of 365 nm, 350 nm is used because there is less interference from the strong 415-nm oxyhemoglobin absorbance. Also, 455 nm is used instead of 450 nm, because Liley intended to use the maximum bile pigment absorbance, and in amniotic fluid we observe that the maximum absorbance is usually much nearer 455 than 450 nm. The baseline is estimated by assuming that it follows a logarithmic curve between 350 nm and 550 nm. The baseline absorbance is then determined at 455 nm and subtracted from the observed sample absorbance at this wavelength. This result, reported as \( \Delta A_{445} \), is usually interpreted by use of a chart given by Liley (1, 2), which he constructed empirically from his own data. The baseline is usually estimated graphically by use of semilog paper; absorbance is plotted on the logarithmic axis vs. wavelength on the linear axis. The entire calculation may be expressed mathematically as

\[
\Delta A_{445} = Y - \exp \left[ .475 (\ln X - \ln Z) + \ln Z \right]
\]

or

\[
\Delta A_{445} = Y - \exp \left[ .475 \ln X + .525 \ln Z \right]
\]

where \( X \) = absorbance at 350 nm, \( Y \) = absorbance at 455 nm, and \( Z \) = absorbance at 550 nm.

Common errors in this determination can be divided into two groups. The first group includes those errors that arise because of sample contamination by materials that absorb appreciably in the 350-550 nm range, the most frequent offender being oxyhemoglobin. While relatively small amounts of oxyhemoglobin can be tolerated if the Liley procedure is used, large amounts render the calculations invalid. These errors, discussed elsewhere (2, 3), will not be commented upon further.

A second group of errors arises from instrumental parameters or from various procedural modifications. These sources of error seem to have been largely neglected in previous discussions of the proper use of this test, although such errors are often substantial. The potential problem is graphically illustrated on the Liley chart in Figure 1. Assume that \( \Delta A_{445} \) of an amniotic fluid sample taken at 35 weeks is correctly determined to be 0.05. Then assume that a second sample taken at 37 weeks has exactly the same composition and that the uncertainty in the method is ±0.01 \( A \). The \( \Delta A_{445} \) obtained for the second sample may thus be anywhere between 0.06 and 0.04. The first extreme, represented by line A in Figure 1, would be interpreted as a very unfavorable trend with a high probability of fetal affection unless an early delivery is undertaken. In contrast, line B would be looked upon as a favorable trend and an early delivery would probably not be considered necessary. It is thus immediately apparent that a
method having an uncertainty of ±0.01 in ΔA455 is insufficiently precise to allow a consistently meaningful interpretation. This paper will discuss three topics that are important in the measurement of ΔA455 and will emphasize how each of these can affect the result: (a) propagation of photometric imprecision, (b) methods of baseline estimation, and (c) the zero absorbance baseline.

Propagation of Photometric Imprecision

In general, propagation-of-error formulas seem to be rather poorly understood and little used in clinical chemistry, although rigorous treatments of this important topic are available (4). For our purpose it is sufficient to inquire about the maximum error to be expected in a single measurement of ΔA455, given estimates of error for the component variables. Manufacturers’ specifications for the uncertainty in photometric precision are usually given in terms of limits of error, that is, the total range within which the quantities can be expected to fluctuate. The problem of interest is to examine how these uncertainties are propagated in equations such as Equation 1 and to arrive at an expected uncertainty in the final value. One theoretical approach to the problem will be given and some experimental results will be discussed. Since the measurement process under discussion involves single, not repetitive, measurements of absorbance at the various wavelengths of interest, the errors are propagated as systematic errors. In general, for any function of three independent variables, F = f(a,b,c), a bound for the systematic error in F is given by (4)

\[ ΔF = \left| \frac{∂F}{∂a} \right| Δa + \left| \frac{∂F}{∂b} \right| Δb + \left| \frac{∂F}{∂c} \right| Δc \]

In other words, this expression related the limits of uncertainty in the measured quantities, Δa, Δb, and Δc, to the limit of uncertainty, ΔF, in the calculated results. The absolute values of the terms are used because the limit of error calculation seeks the worst case ΔF, i.e., that case in which the component errors reinforce each other and maximize ΔF. To see how this applies to amniotic fluid pigment measurements, the procedure is applied to Equation 1, which yields

\[ Δ(ΔA_{445}) = \left( \frac{.475}{X} e^a \right) ΔX + \left( \frac{.525}{Z} e^Z \right) ΔZ + ΔY \]

where \( α = (.475 \ln X + .525 \ln Z) \)

A conservative estimate of the uncertainty in photometric precision for a typical spectrophotometer used in the clinical laboratory is ±0.005 A, although the values quoted by different manufacturers of course vary somewhat. The uncertainty may be significantly greater than this in simple colorimeters. Using a limit of error in each absorbance reading of 0.005 A and recognizing that both a baseline absorbance and a sample absorbance must be measured at each of the three wavelengths, the uncertainties ΔX, ΔY, and ΔZ become 0.01 A. Now the calculated uncertainty in the final result, Δ(ΔA445), may be obtained for any particular set of absorbance readings. Thus, for the typical values of A450 = 0.200 and A445 = 0.020, Δ(ΔA445) is 0.027. For a typical case where ΔA445 = 0.05, an uncertainty of ±0.027 A represents a possible error of 54%, a situation similar to the one discussed previously and illustrated in Figure 1.

Other interesting results are predicated by Equation 2. For example, Δ(ΔA445) is independent of the amount of bile pigment in the specimen, because Y does not appear in the equation. Conversely, the presence of X and Z in Equation 2 indicates that Δ(ΔA445) is sensitive to these two quantities, which primarily reflect the total protein and turbidity in the specimen. Moreover, the absorbance uncertainties at the three wavelengths do not contribute equally to the final uncertainty. While the uncertainty in A445, ΔY, is directly transmitted, the other two terms in Equation 2 involve ΔX/X and ΔZ/Z, respectively. Since Z is almost always much smaller than X, the term ΔZ/Z should be much more important than ΔX/X in determining the final uncertainty. In other words, a change in Z is predicted to affect ΔA445 to a much greater extent than an equal change in X, and one should observe that the answer is affected more by variations in the reading at 550 nm than in one at 350 nm.

Experimental Procedure

A simple set of experiments was performed to confirm the above predictions. Pure bilirubin (Pfanstiehl Laboratories, Waukegan, Ill. 60085)
and human serum albumin were added to a phosphate buffer (0.066 mol/liter) at pH 7.4 to obtain final concentrations of 1.3 mg of bilirubin and 12 g of albumin per liter. This solution has an absorbance spectrum in the visible region which approximates that of a typical amniotic fluid specimen. Absorbances were measured with both a Perkin-Elmer Model 202 (Perkin-Elmer Corp., Norwalk, Conn. 06852) and a Gilford Model 2000 (Gilford Instruments, Oberlin, Ohio 44074) spectrophotometer with 3 ml of the above solution in a standard 1-cm quartz cuvet. The procedure used for the Model 202 was to scan the 350–750 nm region with phosphate buffer in both sample and reference beams. Immediately after this, the bilirubin–albumin solution was placed in the sample beam and the spectrum was scanned again. Absorbances of the bilirubin–albumin solution at the wavelengths of interest were obtained by subtraction. For the Model 2000 spectrophotometer, the zero absorbance level was set on phosphate buffer, the absorbance of the bilirubin–albumin solution was measured, and the zero setting was rechecked. This procedure was repeated for each of the three wavelengths of interest. A blue filter was used at 350 nm with the Model 2000 instrument, to decrease stray light. The above procedures were repeated 10 times on each spectrophotometer, with fresh aliquots of bilirubin–albumin solution being used each time. The solution was protected from direct light as much as possible during the measurement.

Table 1 summarizes the results obtained from the repetitive absorbance measurements on the two spectrophotometers. The absorbance values at 455 nm have been adjusted to correct for an observed change caused by bilirubin decomposition. The rate of such change was 0.004 \( \Delta \) per hour under the conditions described. Also included in Table 1 are the values of \( \Delta A_{455} \) as calculated from Equation 1 and standard deviations for absorbance readings at each wavelength and for \( \Delta A_{455} \) values.

**Table 1. Replicate Measurements of Bile Pigment Concentrations in a Synthetic Amniotic Fluid**

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<th>Model 202</th>
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<td>455 nm</td>
<td>550 nm</td>
<td>( \Delta A )</td>
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<td>.182</td>
<td>.028</td>
<td>.0951</td>
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<td>.180</td>
<td>.028</td>
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\( \bar{X} = .304 \quad SD = .00289 \quad 3 SD = .00870 \)

<table>
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<td>( \Delta A )</td>
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<td>.323</td>
<td>.166</td>
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</table>

\( \bar{X} = .322 \quad SD = .00056 \quad 3 SD = .00870 \)

**Discussion**

First, it is of interest to compare the observed uncertainty in \( \Delta A_{455} \) with that predicted by Equation 2 for the two spectrophotometers. Recall that Equation 2 was derived for a "limit of error" treatment, where the limit of error is defined as the maximum expected deviation from the mean value. Because this concept is not embodied in the common probabilistic treatment of random errors, in this discussion the limit of error in photometric precision at each wavelength is taken as the precision at the 99.7% confidence level, which is three standard deviations. For \( X, Y, \) and \( Z \) in Equation 2, the means of the measured absorbances at each wavelength are used.

Thus, for the data obtained on the Model 2000 spectrophotometer we have

\[
X = .322 \quad \Delta X = .0016
\]

\[
Y = .166 \quad \Delta Y = .0017
\]

\[
Z = .019 \quad \Delta Z = .0019
\]

When substituted in Equation 2 these values yield \( \Delta(\Delta A_{455}) = .006. \) This may be compared with the observed precision, expressed as three standard deviation, which is .004. Likewise, for the data from the Model 202 spectrophotometer we have

\[
X = .304 \quad \Delta X = .0087
\]

\[
Y = .181 \quad \Delta Y = .0080
\]

\[
Z = .028 \quad \Delta Z = .0055
\]

With Equation 2, \( \Delta(\Delta A_{455}) = .018, \) while the observed precision at three standard deviations is \( \Delta(\Delta A_{455}) = .010. \)

The limit of error as calculated from Equation 2 appears somewhat pessimistic in both cases. The
major reason is probably the fact that Equation 2 is strictly valid only if the errors are completely independent. This is probably not the case, because it is reasonable to expect that the factors introducing an error in $A_{455}$ may affect $A_{445}$ similarly, and that the two errors tend to offset each other. Nevertheless, it is clear that the imprecision resulting from the propagation of multiple errors is significantly greater than the instrument specifications for imprecision, which apply only to replicate measurements of a single absorbance.

Another item of interest is to verify the prediction made earlier that the absorbance at 550 nm is much more critical in the calculation of $\Delta A_{455}$ than the absorbance at 350 nm. This result is not obvious, for it might appear that the two values should have equal weight in the calculation; however, the logarithmic transformation results in unequal weighting. The data in Table 2 show values of $\Delta A_{455}$ calculated from different sets of absorbance data.

The fourth column shows the results of $X$, $Y$, and $Z$ having been increased, in turn, by 0.010. It is clearly seen that the increment in $X$ results in a decrease of only 0.001 in $\Delta A_{445}$, while the same change in $Z$ decreases $\Delta A_{455}$ by 0.014.

**Methods of Baseline Estimation**

Since the publication of the Liley procedure, several modifications of the method have appeared. An example, which has appeared in several places (5–7), is the practice of abandoning the semilog absorbance plot of Liley and approximating the baseline by drawing a straight line tangentially to the absorbance curve, as shown by the dashed line in Figure 2. The dotted line represents the baseline obtained by using the logarithmic approximation of Liley. It is evident that $[a/(a+b)]$ is the error introduced by using the linear baseline. The term “error” is used in this context because, when the linear baseline has been used, the resulting $\Delta A$ has still been interpreted by use of the chart given by Liley. This chart represents a correlation of clinical data with values of $\Delta A$ obtained by his method, so the correlation can be expected to be valid only if the method of calculating $\Delta A$ does not differ in any significant respect from the original. The error introduced by using a linear baseline is shown graphically in Figure 3 as a function of $A_{455}$. Again, an $A_{350}$ of 0.200 and an $A_{500}$ of 0.020 have been chosen, because these represent typical values. The practice of using a linear baseline approximation has gained some acceptance because it is operationally much simpler than transferring absorbance readings to semilog graph paper to estimate the baseline. In our laboratory this procedure has been circumvented by making all calculations with a programmed version of Equation 1 on an Olivetti Underwood Model P101 calculator (Olivetti Underwood Corp., New York, N. Y. 10016). This has the obvious advantage of eliminating the errors that may creep in during the process of plotting points on graph paper, constructing the baseline, and going through the several arithmetic steps.
The Zero Absorbance Baseline

A final topic of importance is the necessity of establishing a zero absorbance baseline before making absorbance measurements on the amniotic fluid specimen. This point is probably commonly neglected because no mention has been made of zeroing the instrument against a suitable reference in several previous articles dealing with the method (1–3, 5–8). Indeed, it might at first glance seem that this step is unnecessary, because an artificial baseline is calculated in the course of the data analysis. The problem stems from some peculiar properties of the logarithmic baseline. If all the points on a particular amniotic fluid scan are displaced along the absorbance axis by a fixed amount, the curvature of the logarithmic baseline changes, and a different value of $\Delta A_{450}$ is obtained. An example of this effect is shown in Figure 4, where the error introduced by baseline offset is plotted against the amount of offset. The initial conditions chosen were $A_{450} = 0.200$, $A_{450} = 0.120$, and $A_{450} = 0.020$. This curve illustrates the size of the error that can arise from baseline offset, although the percent error also depends on $A_{450}$ becoming somewhat smaller as $A_{450}$ increases. Note that baseline errors of this magnitude could be caused by measuring the sample against air instead of a reference solution, due to absorption and scattering of radiation by the cuvet and the solvent. Clearly, a uniform practice is necessary regarding the zero baseline, and it is recommended that all absorbance measurements be referenced against pure water in a matched cuvet.

In summary, some of the uncertainties in amniotic fluid bile-pigment determinations arising solely from the spectrophotometer or from improper procedures have been discussed. It has been demonstrated that the magnitude of these errors can easily invalidate the interpretation of this important test and may mislead the clinician. Three suggestions are offered for minimizing the errors in this procedure. First, the spectrophotometer employed should have very high photometric precision and should be readable to 0.001 A. Second, the calculation of $\Delta A_{450}$ should be entirely carried out with a computer or programmed calculator to avoid those errors that would be introduced in a manual graphical calculation. Third, the spectrophotometer must be properly zeroed and the logarithmic baseline estimation used. It is suggested that amniotic fluid measurements that are so optimized might result in a higher correlation of $\Delta A_{450}$ with fetal distress than has been reported to date.

References