mg/liter, falsely positive results may occur. Over the past year we have seen 13 cases in which the direct bilirubin was 25-30 mg/liter, but there were no false positives. A false positive might be predicted by setting up the complete test as described in the "time" paragraph above. A true positive should be positive in all tubes. A borderline or mildly positive test should show a prominent increase in color intensity in the tubes with added bilirubin. If this is not the case, a false positive should be suspected.

References

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Inadequate Sample-Preparation Technique As a Source of Error in Determination of Erythrocyte Folate by Competitive Binding Radioassay

To the Editor:

Freezing and thawing is an established method of inducing hemolysis of whole blood before folate is determined by either the classical biological method or competitive binding radioassay. Nevertheless, some manufacturers of radioassay kits for the analysis of folate recommend that whole blood be diluted with ascorbic acid solution and left at room temperature to produce hemolysis. We have tried two such kits (Diagnostic Products Corporation and Bio-Rad) and obtained evidence that these procedures yield suboptimal values for erythrocyte folate.

The suspicion arose that hemolysis was incomplete after treatment with ascorbic acid (10 g/liter solution) when we analyzed fresh blood from 16 healthy volunteers and repeated the analysis after the samples had been stored at -20 °C for two weeks. The concentration of folate in serum was unchanged after storage, but the concentration in whole blood that had been frozen was 106.4 ± 6.4 μg/liter (mean ± SEM) as compared to 97.1 ± 6.7 in fresh blood (Diagnostic Products Corporation), a statistically significant (P < 0.01, Wilcoxon paired sample test) increase of 8.7%.

We next did experiments with both kits, using the recommended procedures of sample preparation with fresh blood and with blood that had been frozen and thawed repeatedly. Results were invariably 10 to 17% higher in the samples that had been frozen and thawed before incubation with ascorbic acid.

The dilution of whole blood before analysis was also found to influence the results. We tried different dilutions with the ascorbic acid solution in the range of eight- to 64-fold and compared the results to the values for whole blood folate found when the recommended (Diagnostic Products Corporation) dilution of 21-fold was used. The calculated concentration increased with increasing dilution from eight- to 32-fold, but no further change at 64.

We confirmed the effect of dilution in a control experiment, using a different blood sample that had been frozen and thawed repeatedly. Aliquots of this sample were then diluted 21-fold (n = 17) or 32-fold (n = 16) and then left to stand and treated as recommended by the manufacturers. The concentration of folate in whole blood was found to be 103.0 ± 1.7 μg/liter (mean ± SEM) and 113.7 ± 3.6 μg/liter for the former and the latter dilutions, respectively (P < 0.01, Mann-Whitney test).

We have not done experiments to establish the mechanism of the effect of dilution on the radioassay method. However, this phenomenon was observed both with fresh blood and with samples that had been frozen and thawed repeatedly, and thus factors other than hemolysis and membrane rupture are probably involved. Possibly blood constituents interfere in some way with the folate/ligand interaction and this effect becomes less significant at higher dilutions.

To study the effect of temperature and to establish an alternative sample preparation method, we treated aliquots of fresh blood from a healthy volunteer as summarized in Table 1. The aliquots were either frozen (acetone/solid CO2) and thawed once, twice, or three times, diluted to 32-fold with ascorbic acid solution and further processed immediately or they were diluted and left to stand for 90 min at 4, 22, or 37 °C before analysis.

The highest concentration (207.2 ± 5.1 μg/liter) was found in the aliquots that had been frozen and thawed once. The values decreased when this procedure was repeated twice or three times. Again, leaving the samples to stand at room temperature (22 °C) resulted in lower values (119.2 ± 22.5 μg/liter) and still lower ones at 37 °C. The yield improved considerably at 4 °C as compared to the experiments carried out at the higher temperatures, but the mean value was considerably lower than that obtained with the freezing and thawing method.

Because the yield of folate in the first series of experiments was increased by subsequent freezing and thawing of samples that had been left to stand with ascorbic acid, it appears that the incubation method is not sufficiently efficient in producing rupture of the erythrocyte membranes and making folate available for reaction with the ligand. The findings reported in Table 1 also suggest temperature-dependent degradation of folate during incubation. The yield of folate by the procedure recommended by the manufacturers was 42.5% lower than that obtained when the samples were frozen and thawed once.

We conclude that the established method of freezing and thawing should be used instead of the incubation procedures. We have also found it advantageous to dilute whole blood more than recommended by the manufacturers.

Table 1. Whole-blood Folate Concentration in a Healthy Volunteer, As Determined by Radioassay after Different Methods of Sample Preparation

<table>
<thead>
<tr>
<th>Fresh blood (EDTA anticoag.) diluted</th>
<th>Folate found, μg/liter</th>
</tr>
</thead>
<tbody>
<tr>
<td>32-fold with ascorbic acid soln.</td>
<td></td>
</tr>
<tr>
<td>Left to stand at 4 °C for 90 min (n = 14)</td>
<td>147.2 ± 3.6*</td>
</tr>
<tr>
<td>Left to stand at 22 °C for 90 min (n = 15)</td>
<td>119.2 ± 2.2</td>
</tr>
<tr>
<td>Left to stand at 37 °C for 90 min (n = 15)</td>
<td>98.4 ± 2.6</td>
</tr>
<tr>
<td>Frozen and thawed once (n = 15)</td>
<td>207.3 ± 5.1</td>
</tr>
<tr>
<td>Frozen and thawed twice (n = 15)</td>
<td>188.3 ± 5.8</td>
</tr>
<tr>
<td>Frozen and thawed three times (n = 14)</td>
<td>173.0 ± 3.9</td>
</tr>
</tbody>
</table>

* Mean ± SEM

CLINICAL CHEMISTRY, Vol. 23, No. 8, 1977 1505
Interlaboratory Variability in Amylase Assays

To the Editor:

For the Phadebas Amylase Test (Pharmacia), now in quite widespread use as a convenient and relatively precise method for the estimation of plasma or serum amylase activity, the makers claim a CV of not more than 6% for all measurable activities. Melnychuk (1) achieved a CV of 2.8% and Rosalki and Tariow (2) one of about 2%. In our laboratory, we found a CV of about 4% over three separate batches of the reagent tablets for control sera with activities ranging from 290 to 420 U/liter. This precision is considerably better than that obtained with the more conventional procedure of Street and Close (3), with which we found CV’s of 7.1% to 9.9% with use of the same sera.

In spite of these relatively good CV’s within individual laboratories, between-laboratory precision is disappointing. The fortnightly reports of the Wellcome Group Quality Control Programme (Wellcome Research Laboratories, Beckenham, England) show the CV for the Phadebas procedure to be about 18%, as compared with about 25% for all methods. Although individual laboratories may be capable of performing the Phadebas test with precision, they perhaps may be consistently inaccurate as a result of deviation from scheduled procedure or incorrect calibration. For instance, the calibration curve supplied with each batch of reagent tablets may not apply to the user’s particular laboratory conditions, and quality of the spectrophotometer employed could be important in this respect.

Phadebas Reference Serum, which has an assigned Phadebas amylase value, can be used to give a single point calibration check at a normal value for plasma, or at other activities by varying the duration of incubation. However, reference sera with higher activities would enable the user to construct his own calibration curve under his own laboratory conditions. In fact, Pharmacia has recently produced such a serum, but it is not yet commercially available. Use of such sera should ensure close agreement of test results between customer and manufacturer. Nevertheless, the accuracy of these results entirely depends on the reliability of the standardization procedure adopted by Pharmacia, which uses Hyland Multi-Enzyme Reference Serum as a source of amylase and reassay this by the same saccharogenic method that Hyland use to assign values initially (4), but perform the estimation at 37 °C. Hyland supply amylase values for their reference sera by both the saccharogenic method at 40 °C and the Phadebas method at 37 °C, and theoretically the Phadebas results should be lower by about 13% (5). However, this is not always so (Table 1) and may indicate discrepancies in saccharogenic results between the two manufacturers or differences in Phadebas technique. Another complication stems from the fact that Hyland sera contain porcine α-amylase, and α-amylase from other than human sources may behave differently in the Phadebas test (6).

The statement by Pharmacia that their calibration curve is based on Hyland Multi-Enzyme Reference Serum may imply to the customer that they are conferring approval on assigned values for this serum. If this assumption is made and Hyland serum is used to reconstuct the curve, then a bias in results may occur relative to those which would be obtained by Pharmacia.

In summary, unless one sets up a reference method in one’s own laboratory, one is dependent on the accurate calibration of the method by Pharmacia, and strict adherence to scheduled conditions and technique is necessary to minimize errors (7). Phadebas Reference Serum gives a valuable calibration check under the customer’s laboratory conditions, but cannot be regarded as a primary standard. However, the use of Hyland or other commercially available sera for this purpose may result in deviation from the initial Phadebas calibration, and it is not easy for the user to decide which is correct. All these factors could lead to poor agreement among laboratories using the Phadebas Amylase Test, in spite of good precision within individual laboratories.

References


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A representative of Pharmacia responds:

To the Editor:

We at Pharmacia agree that variability between laboratories should be a concern.

To reduce such between-laboratory variability, we agree that a control must be used. Accordingly, since Pharmacia has recognized this need, it has recently made available Phadebas ACR (Amylase Calibration Reference Serum). This product allows for calibration of the Phadebas Amylase Test. It is a human-based serum product that contains a porcine supplement, which must be used because of the unavailability of a sufficient supply of human amylase. How-

Table 1. Examples of Manufacturer’s Assigned Values for Hyland Multi-Enzyme Reference Sera

<table>
<thead>
<tr>
<th>Reference serum</th>
<th>Saccharogenic (U/liter) at 40 °C</th>
<th>Phadebas (U/liter) at 37 °C</th>
</tr>
</thead>
<tbody>
<tr>
<td>A, Lot 3055X004A1</td>
<td>167</td>
<td>186</td>
</tr>
<tr>
<td>B, Lot 3056X004A1</td>
<td>749</td>
<td>840</td>
</tr>
<tr>
<td>C, Lot 3057L001AA</td>
<td>1215</td>
<td>1245</td>
</tr>
</tbody>
</table>

1508 CLINICAL CHEMISTRY, Vol. 23, No. 8, 1977