Simplified Photometric Copper-Soap Method for Rapid Assay of Serum Lipase Activity

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We modified the method of Yang and Biggs [Clin. Chem. 17, 512 (1971)] for greater speed and simplicity. Serum is incubated with an emulsified olive oil substrate, the fatty acids extracted, the copper soap formed, and phases separated, all in a single tube. An aliquot of the organic phase is removed for color development. One analysis can be completed in 35 min, 20 in less than 2 h. The method is accurate and precise. Results correlate well with the unmodified colorimetric method, and with the classical titrimetric lipase procedure. The reference interval (normal range) is 20 to 180 U/liter.

Additional Keyphrases: pancreatic disease • intermethod comparison • normal values (reference interval)

We have modified the Yang and Biggs method so that serum/enzyme incubation, fatty acid extraction, copper soap formation, and phase separation are all done in the same glass test tube, thus eliminating any aspiration and phase transfer steps. An aliquot of the organic (upper) phase is removed for final color development. The result is a much faster, more practical, and precise method, with fewer opportunities for error.

Materials and Methods

Reagents

The emulsified olive oil substrate and several of the following reagents are substantially as described by Yang and Biggs (8). All solvents and chemicals are analytical grade.

Tris buffer, 57 mmol/liter, pH 8.0. Dissolve 6.9 g of tris(hydroxymethyl)aminomethane in about 950 ml of water. Adjust the pH to 8.0 with about 30 ml of 1 mol/liter HCl and dilute to 1.0 liter with water.

Acacia solution. Dissolve 30 g of acacia (gum arabic powder; Matheson, Coleman and Bell, Norwood, Ohio 45212) and 0.4 g of sodium benzoate in about 150 ml of water. Dilute the mixture to 200 ml and filter through glass wool to remove insoluble substances. Store in refrigerator.

Purified olive oil. Firmly pack glass wool to a height of about 6 cm in a 3.5 × 45 cm glass chromatography column. Add 80 g of neutral aluminum oxide (Woelm, Eschwege, W. Germany; Activity Grade I) and 200 ml of high-quality olive oil (we used Bertolli Pure Olive Oil, Lucca, Italy; or Matheson, Coleman and Bell, purified olive oil). Connect the bottom of the column to a vacuum flask attached to a water aspirator to expedite chromatography.

Stock olive oil emulsion. Add 90 ml of purified olive oil and 210 ml of the acacia solution to a glass homogenizer (Waring Blender; Waring Products Co., New Hartford, Conn. 06057). Refrigerate for at least 30 min, then homogenize for 3 min at low speed and 10 min at high speed. Refrigerate for 30 min or more and repeat the 10-min high-speed homogenization. Store in refrigerator and mix well just before using. Refrigerated, this mixture is stable for several months.

Working olive oil emulsion. Measure three volumes of Tris buffer and two volumes of stock olive oil emulsion into a graduated cylinder. Produce a homo-
geneous mixture by about 30 s of vigorous shaking by hand. (Prepare this freshly each day and refrigerate between uses.) Shake well before each use.

**Extraction solution.** Mix three volumes of chloroform with two volumes of heptane.

*Stock oleic acid standard solution, 6.0 mmol/liter.* Add 169.5 mg of oleic acid (Schwarz/Mann, Orangeburg, N.Y. 10962) to a 100-mL volumetric flask. Dissolve and dilute to volume with the extraction solution. Store refrigerated in a tightly capped bottle.

*Working oleic acid standard solution, 60 μmol/liter.* Dilute 1.0 mL of the stock standard solution to 100 mL with extraction solution in a volumetric flask, and store refrigerated, in a tightly capped bottle.

*Copper reagent, pH 8.3.* Place 50 mL of 1 mol/liter acetic acid (57.3 mL of glacial acetic acid per liter of distilled water), 85 g of triethanolamine (Eastman Kodak Co., Rochester, N.Y. 14650), and 23.75 g of cupric nitrate trihydrate into a 1-Liter beaker. Add about 600 mL of distilled water and dissolve with the help of a magnetic stirrer. Add 250 g of sodium chloride and dissolve. Dilute the mixture to about 975 mL, adjust the pH to 8.3 with a few drops of glacial acetic acid or triethanolamine, and dilute to volume with water. Keep tightly capped. Discard if a green precipitate forms. This reagent is stable for several months at room temperature.

*Bovine serum albumin solution, 20 g/liter.* Dissolve 50 mg sodium azide and 7.0 g of bovine serum albumin solution (Cohn Fraction V, 300 g/liter solution; Nutritional Biochemicals, Cleveland, Ohio 44129) in a 100-mL volumetric flask and dilute to volume with the Tris buffer. Store refrigerated; discard if insoluble material appears.

*Color reagent.* Add 100 mg of sodium diethyldithiocarbamate to 60 mL of n-butanol in a 100-mL volumetric flask and dilute to volume with chloroform. This reagent is stable for at least two weeks in the refrigerator. Warm a sufficient amount to room temperature before using.

*Saturated sodium bromide solution.* Add a minimum of 510 g of sodium bromide to 500 mL of distilled water. Mix with magnetic stirring for about an hour. Let the mixture stand overnight, then filter through a pad of glass wool. Store at room temperature.

**Procedure**

Use acid-washed tubes and pipets throughout.

1. Add 1.0 mL of working olive oil emulsion to four culture tubes1 (16 X 125 mm) with Teflon-lined screw caps. Label them “Sample,” “Sample Blank,” “Standard,” and “Reagent Blank.”

2. Place Sample and Sample Blank in a 37 °C water bath for about 3 min.

3. Add 100 μL of serum to the Sample tube. Mix briefly with a vortex-type mixer and return to the water bath. (Repeat this step at 30-s intervals for any additional samples.)

4. After exactly 10 min, add 7.0 mL of extraction solution to the sample, cap tightly, and shake vigorously for several seconds. (Repeat this step at 30-s intervals for any additional samples.)

5. Add 7.0 mL of the extraction solution to the Sample Blank, followed by 100 μL of serum. Cap immediately and shake vigorously for several seconds. (Repeat for any additional sample blanks.)

6. Add 7.0 mL of extraction solution to the Reagent Blank tube.

7. Add 7.0 mL of the working standard solution to the Standard tube. Cap the tubes tightly. All subsequent steps are followed as a batch process.

8. Shake all tubes for 4 min with a mechanical shaker at high speed, then add 1.0 mL of the saturated sodium bromide solution and 3.5 mL of the copper reagent to each tube.

9. Cap tubes and shake for 2 min with a mechanical shaker at low speed. Centrifuge the tubes for 10 min at 1600 X g or greater in a swinging bucket rotor.

10. Transfer 3.0-mL aliquots of each organic layer (upper phase) into appropriately labeled test tubes or colorimeter tubes, and cap the tubes containing the remainder in case the test must be repeated.

11. Add 0.5 mL of the color reagent to each tube and mix briefly on vortex-type mixer. Read absorbances at 435 nm of the standard (A<sub>s</sub>), sample (A<sub>x</sub>), and sample blank (A<sub>xB</sub>) vs. the reagent blank.

If the absorbance of the sample exceeds that of the standard by more than 20%, either make appropriate dilutions with extraction solution and reread both sample and sample blank, or repeat steps 10 and 11 with appropriate dilutions of the organic extract in the culture tube. Do not dilute the standard. If the calculated lipase value exceeds 4000 U/liter, prepare an appropriate dilution of the serum with bovine serum albumin and repeat steps 1 through 11.

**Calculation**

\[
U/\text{litter} = \frac{A_x - A_{xB}}{A_s} \times 0.42 \mu\text{mol} \times \frac{1000 \text{ mL}}{0.10 \text{ mL}} \times 10 \text{ min}
\]

Simplified, this is

\[
U/\text{litter} = \frac{A_x - A_{xB}}{A_s} \times 420
\]

where U is defined as 1 μmol of fatty acid liberated per minute at 37 °C, 0.42 μmol = amount of oleic acid in 7.0 mL working standard solution, 0.10 mL = volume of sample, 1000 mL = volume correction factor, and 10 min = incubation time.

**Results and Discussion**

**Analytical Variables**

Single-tube procedure. Simplification of the copper soap lipase assay has four essential requirements: (a) the final chloroform/heptane fatty acid extract

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1 Kimble Products, Owens-Illinois, Inc., P.O. Box 1035, Toledo Ohio 43666. Cat. No. 73750 (disposable), or cat. No. 45066A.
must form the upper phase to avoid sporadic errors as a result of copper reagent contamination when aliquotting; (b) emulsion formation in the organic extract must be prevented; (c) organic and aqueous phases must be cleanly separated; and (d) the sensitivity of the procedure must be maintained. The phases can be reversed by adding a sufficient amount of water-soluble solute to the aqueous phase to give it a density greater than that of the chloroform/heptane extraction solution. Any of the highly water-soluble salts of Li, K, or Na, as well as ammonium sulfate, cupric nitrate, urea or sucrose, increase the density of the aqueous mixture of olive oil emulsion and copper reagent sufficiently to cause the chloroform/heptane extraction solution to become the upper phase, but only one solute, NaBr, satisfied all three remaining requirements, particularly to prevent emulsion formation without a large loss in sensitivity. Emulsion formation was particularly difficult to overcome. Even in the presence of NaBr, an emulsion can form if high-speed shaking is excessive.

Copper reagent. Yang and Biggs (8) increased the sensitivity of the copper-soap technique by adding NaCl to the copper reagent. However, we found the near-saturation amount of NaCl and the pH specified produced a very unstable reagent. We decreased the NaCl content, and increased the pH from 8.1 to 8.3 with triethanolamine to produce a reagent that is stable for several months at room temperature. The small decrease in sensitivity resulting from these modifications is offset by the addition of saturated NaBr at a subsequent step in the procedure.

Color reaction. The color produced with diethylidithiocarbamate is linearly related to oleic acid concentration to about 75 μmol/liter, a concentration that produces net absorbances of about 0.85 in the Cary 15 spectrophotometer or 0.65 in the Dow filter photometer. This is equivalent to enzyme activities exceeding the upper limit of the normal range by more than 2.5-fold. Greater absorbances may be brought within the linear portion of the curve by simply diluting the final color, or by appropriately diluting the organic extract with extraction solution before the color reagent is added. The color is stable for at least 15 h.

Another copper-chelating reagent, 1,5-diphenylcarbazide (9), a considerably more sensitive color reagent, has been used in several free fatty acid methods. However, we found this reagent to be unsuitable both because of the length of time required for color development and the brief color stability, which varied significantly from sample to sample.

Optimal pH. pH's ranging between 7 and 9 have been used in lipase assays. We found that normal sera generally show a pH optimum that varies between 7 and 7.6. However, sera with abnormal lipase activity display broad pH activity curves, with optimal activity ranging between pH 7.5 and 8.5. A substrate pH of 8.0 was chosen as a suitable compromise for accurately assaying any lipase activity.

Substrate reproducibility. Stability and reproducibility of emulsion preparation have been major areas of concern in methods in which emulsified substrates are used. We found that the stock olive oil emulsion reported by Yang and Biggs (8) is stable for several months. More importantly, we found the reproducibility of this substrate to be excellent from batch to batch (Table 1). Thus nonreproducible substrate preparation and substrate instability are not problems in this method.

### Linearity Studies

**Linearity with respect to time.** In contrast to previous reports (5, 8), we found this enzymatic reaction to be linear for only 10 to 15 min, whether lipase activities were normal or abnormal (Figure 1). Sometime between 10 and 15 min there is a change to a slower linear reaction. During the 10-min incubation we used in the method, however, the enzyme reaction follows zero-order kinetics.

**Linearity with respect to enzyme.** Sera with highly abnormal lipase activity must be diluted to avoid ex-

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**Table 1. Reproducibility of Olive Oil Emulsion Substrate**

<table>
<thead>
<tr>
<th>Substrate batch</th>
<th>Lipase activity&lt;sup&gt;b&lt;/sup&gt; of serum pool, U/liter</th>
</tr>
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<tbody>
<tr>
<td>No. 1</td>
<td>298</td>
</tr>
<tr>
<td>No. 2</td>
<td>303</td>
</tr>
<tr>
<td>No. 3</td>
<td>299</td>
</tr>
<tr>
<td>No. 4</td>
<td>298</td>
</tr>
</tbody>
</table>

<sup>a</sup> Each batch includes independent preparation of purified olive oil, stock olive oil emulsion, and working olive oil emulsion.

<sup>b</sup> Average of duplicate determinations. All batches were assayed together in a single run.

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**Fig. 1. Linearity of enzyme reaction with respect to time**

Sera from a healthy subject (activity, 120 U/liter) and a patient with pancreatitis (activity, 2900 U/liter) are compared.
ceeding the desired conditions of saturating substrate concentrations. Figure 2 shows the linearity of the reaction with respect to enzyme concentration. When decreasing amounts of enzyme were produced by decreasing the volume of serum assayed, we found that there is usually a disproportionate decrease in enzyme activity as serum volume is decreased to volumes <50 μl. Inactivation of the enzyme on this account can be avoided if the serum is diluted with an appropriate diluent before the assay. Dilution of the serum with water, physiological saline, or heat-inactivated (45 min at 60 °C) serum did not prevent this loss of activity. Bile salts and bovine serum albumin have previously been shown (3, 10) to prevent irreversible inactivation of lipase. We confirmed that sodium deoxycholate (2 nmol/liter) will prevent a loss of lipase activity, but such bile salts produce an unacceptable high blank with the copper-soap method. Albumin in concentrations ranging between 20 and 70 g/liter also protects the lipase from inactivation, but without producing a large increase in the blank value. As shown in Figure 2, dilution of an abnormal serum with a solution of bovine serum albumin (20 g/liter) results in a reaction that is completely linear with respect to enzyme activity up to about 4000 U of lipase activity per liter, above which there is a slow deviation from linearity. Thus, lipase activities up to 22-fold the upper limit of the normal range may be assayed before a dilution of the serum with albumin solution is required.

Fatty Acid Recovery

When the oleic acid working standard is carried through the extraction procedure, the resulting absorbance averages 83% (SD, ±2.8%) of that produced by a standard not carried through the extraction. To correct for such losses, the working standard is routinely processed like a sample. Fatty acid recovery at other concentrations was determined by adding 150, 300, and 600 nmol of oleic acid to portions of a serum pool containing 91.5 nmol of endogenous fatty acid per milliliter. Recoveries at all three concentrations ranged from 97 to 102%.

Potential Interferences

Hemoglobin reportedly either inhibits lipase activity (11, 12) or has no effect (3). Hemoglobin standard solution (Dow Diagnostics, Dow Chemical, Indianapolis, Ind. 46225) was added to a serum pool with slightly increased (390 U/liter) lipase activity. Final concentrations of hemoglobin up to 1200 mg/liter had no effect on lipase activity. Bilirubin, added to serum with increased (1100 U/liter) lipase activity also had no effect at concentrations up to 150 mg/liter.

Precision

Between-day precision was determined by 20 consecutive daily analyses of pooled sera that had been divided into aliquots and frozen. For lipase activity in the mid-normal range, the coefficient of variation

Fig. 2. Linearity of reaction with respect to enzyme concentration

The lipase activity of serum from a patient with pancreatitis (activity, 1650 U/liter) is shown with respect to decreasing serum volume. The lipase activity of another abnormal serum (activity, 8400 U/liter) is shown with respect to decreasing enzyme activity produced by diluting the serum with a 20 g/liter bovine serum albumin solution before the assay.

Fig. 3. Results obtained by the proposed method compared with results obtained by (f) the Yang and Biggs (6) method, and (B) the Henry et al. (11) titrimetric method.

(A) 40 samples; y = 1.02x - 8.9 U/liter; standard error of the estimate of the least-squares line = 17.2 U/liter; SD = 21.1 U/liter; x = 398 U/liter; y = 397 U/liter; p-value = 0.269
was 4.4% (mean, 74 U/liter; range, 67 to 79 U/liter). At sixfold the upper limit of normal, the coefficient of variation was 1.3% (mean, 1170 U/liter; range, 1130 to 1180 U/liter).

Method Comparison

Values obtained by the shortened method described here were compared with those obtained by the method of Yang and Biggs (8). The relationship between the two methods is linear, as shown in Figure 3A. Student's t-test and the sign test (13) reveal no difference between results by the two methods. Spearman's coefficient of rank correlation coefficient is 0.99, showing excellent correlation between the two methods. Comparison with the Henry et al. (11) modification of the Cherry and Crandall (4) titrimetric method (Figure 3B) also shows good correlation, albeit nonlinear. The Spearman coefficient of rank correlation coefficient is 0.93. This relationship is highly nonlinear because the titrimetric method does not follow zero-order kinetics. The increasing deviation from linearity with increasing enzyme activity may result from product inhibition in the titrimetric procedure. However, enzyme inactivation, substrate depletion, or a shift from the optimum pH during the 16-h incubation may also contribute. These potential inadequacies are obviated by the short incubation of the proposed method. As a result, the colorimetric method is much more sensitive and accurate, particularly for detecting and measuring abnormally high serum lipase activities.

Normal Range

We assayed sera from 83 presumably healthy adults (42 men, 41 women), all laboratory personnel. The data were not normally distributed. Analysis of the data by nonparametric statistics (14) gives a normal range of 20 to 180 U/liter (mean, 73 U/liter) with no significant sex-related difference. This normal range agrees well with those reported by others (Table 2). Of particular interest is the comparison with the normal range reported by Tietz and Repique (3). The results by this kinetic, titrimetric procedure agree very well with those reported here for the colorimetric copper soap assay.

Our method is both rapid, accurate, and precise, and requires no specialized equipment or skills. As a result, we believe it offers the physician a highly practical and reliable tool for the diagnosis of pancreatic disorders.

References