Interference in a Chromogenic α-Amylase AssayCaused by Dye-Labeled Oligosaccharide-Induced Precipitation of Lipoprotein

Gary G. Wickus, Russell O. Dukerschein, Judy R. Pierce, and Kent D. Davis

We examined the mechanism by which hypertriglyceridemia interferes with the Roche Diagnostics “Amylochrome” procedure for measurement of amylase in lactescent samples with normal and above-normal amylase activity. The serum blank recommended in the Amylochrome protocol to compensate for lactescence remains inappropriately turbid, compared with the test, and is partly responsible for the underestimation of amylase activity. Other major interference is seen when lipoprotein in hyperlipemic samples with above-normal amylase activity reacts with the soluble oligosaccharide–triazinyl dye product of the Amylochrome assay to form a flocculent blue precipitate. The formation of this precipitate in the test, and its removal, diminish lactescence due to lipemia, an effect that cannot be matched by any manipulation of a serum blank procedure. Oligosaccharide–dye product is removed as a component of the precipitate.

Additional Keyphrases: analytical error • hypertriglyceridemia • pancreatitis • lipemia

The association of hyperlipemia with apparently normal serum α-amylase (EC 3.2.1.1) activity in serum of patients with acute pancreatitis has been reported for saccharogenic (1, 2), starch–iodine (3–5), and chromogenic assay methods (6, 7). The high incidence of normal amylase activity has been attributed to direct analytical interference from lipemia (4, 8), to the presence of amylase inhibitors (2), or possibly to a true characteristic of acute pancreatitis with associated lipemia (6, 7). In our laboratory, the Amylochrome chromogenic assay (Roche Diagnostics, Nutley, NJ 07110) for measuring amylase activity gives low results with lipemic serum samples. Inspection of the serum blank prepared according to the manufacturer’s protocol suggested that the blanking procedure is responsible for this underestimation. Here we report our evaluation of an alternative two-step blank procedure for the Amylochrome amylase assay and identify an additional source of analytical interference.

Materials and Methods

The Amylochrome amylase assay was conducted at 37 °C according to the manufacturer’s instructions. Absorbance was measured at 625 nm in a Model 54B spectrophotometer (Perkin-Elmer Corp., Norwalk, CT 06856). Results, expressed as dye-units per 100 mL, were calculated from a calibration curve constructed according to the manufacturer’s instructions. The reference interval for amylase activity in serum is up to 200 dye units/100 mL; for urine it is up to 250 dye units/h.

We used two blank procedures to correct for lactescence in the test assay.

The manufacturer’s recommended blank was prepared by adding the substrate pellet to 1.9 mL of distilled water. This mixture was incubated for 15 min at 37 °C, then 8.0 mL of pH 4.3 diluent and 0.10 mL of serum sample were added to the tube. The blank was centrifuged to remove insoluble substrate, and the absorbance of the supernate was used to correct for lactescence in the test sample.

To construct a two-step blank, the Amylochrome tablet was incubated for 15 min at 37 °C in 1.9 mL of distilled water. The blank tube was centrifuged and only the supernate was added to 0.10 mL of serum in a separate test tube. This mixture was incubated for 15 min at 37 °C. After the second incubation, we added 8.0 mL of the diluent to the mixture and recombined this solution with the insoluble substrate pellet in the original tube. After centrifugation, the absorbance of the supernate was used to correct for lactescence in the test sample. Blanks and tests were read individually against a reagent blank, prepared by heating a tablet for 15 min at 37 °C in 2.0 mL of distilled water, followed by addition of 8.0 mL of the diluent.

We also measured serum amylase activity by using a soluble maltotetraose substrate and coupled enzyme system that produces NADH (9), as formulated by Boehringer Mannheim Diagnostics, Houston, TX 77063. Amylase activity was measured at 37 °C in a Rotochem IIa/36 centrifugal analyzer (Travenol Laboratories, Deerfield, IL 60015) according to the reagent manufacturer’s application for this instrument. The reference interval for this assay was up to 100 U/L. Lipemic specimens were clarified either by leaving them overnight at 4 °C or, when specified, by ultracentrifugation at 143 000 × g for 15 min in a Model L5-75 ultracentrifuge (Beckman Instruments, Inc., Fullerton, CA 92634).

To examine the apparent chromogenic reaction-product-induced precipitation of lipoprotein elements from lipemic serum samples, we generated a reagent blank pool and two pools of soluble oligosaccharide–triazinyl dye product from insoluble substrate by using the standard protocol. The absorbance of pool one was 0.314 and contained soluble dye product equivalent to amylase activity of 310 dye units per 100 mL. Pool two, with an absorbance of 0.786, represents product formation equivalent to amylase activity greater than 620 dye units per 100 mL. The absorbance of the reagent blank pool was 0.00. Dilutions of a clear urine sample or clear serum sample were used as a source of amylase activity. A lipemic serum pool (57.1 g of triglyceride per liter) was used as the source of lipoprotein. Samples from each pool (1.1 mL) were incubated with 10, 25, 50, and 100 μL of lipemic serum for 15 min at 37 °C. Eight milliliters of diluent was added to each tube at the end of the incubation.

Results

We assayed samples with different degrees of lipemia for amylase activity (Table 1). If the unblanked apparent amylase activity was corrected for lactescence with the manufacturer’s recommended blank, low or negative activities were calculated. The two-step blank gave corrected amylase activities that were higher and agreed better with the amylase activity measured in the unblanked clarified portion of each sample. The application of a two-step blank to specimens with normal clarity gave amylase activity that agreed with the unblanked
activity, but use of the manufacturer's blank resulted in a low estimate of amylose activity. In each case, the absorbance of the one-step blank exceeded the absorbance of the two-step blank.

Hyperlipemic samples with normal amylose activity produced no precipitate in the Amylochrome amylose assay.

Extremely lipemic serum samples, obtained from two patients with acute pancreatitis and associated above-normal urine amylose activity, were analyzed for amylose activity with the chromogenic amylose procedure and the soluble maltotetraose amylose assay. A third lipemic sample was prepared by adding an equal volume of a clear serum sample with above-normal amylose activity to a lipemic sample. One portion of each lipemic sample was clarified by ultracentrifugation. The clarified and unclarified samples gave comparable above-normal activity when assayed with the maltotetraose technique (Table 2), and the values remained proportionately unchanged when the samples were diluted. Above-normal amylose activity could also be observed when the clarified samples were assayed by the Amylochrome method, but the original lipemic samples demonstrated much lower activity when the two-step blanking procedure was used to correct for turbidity (Table 2). The one-step blanking procedure was not used to correct for lactescence in these samples, because the absorbance of this type of serum blank was always greater than the test. The Amylochrome assay produces a floculent blue precipitate when above-normal amylose activity is measured in the presence of hypertriglyceridemia. The precipitate that accumulates in the test after addition of diluent does not form in the two-step or one-step serum blank (Figure 1, top view). Removal of blue precipitate from the test by routine centrifugation decreases the lactescence in the infranate of the test but not in the blank (Figure 1, side view).

The prepared pools of soluble oligosaccharide-triazinyl dyes, mixed with lipemic serum, also produced a floculent blue precipitate after addition of diluent. The precipitate formed in tubes from pools one and two but not in the blank pool. The amount of precipitate that formed increased and the density of the precipitate decreased as increasing amounts of lipemic serum were mixed with 1.1-mL samples from pools one and two. The precipitate that formed in samples from pool two could be removed by routine centrifugation except in the tube containing 0.05 mL of lipemic serum; the precipitate in this tube remained suspended. After removal of precipitate by centrifugation, the solutions in the other tubes containing 10, 25, and 100 mL of lipemic serum were clear, with no residual lactescence. The absorbance of the mixture in these tubes was less than the initial absorbance of pool two, and the decrease in the absorbance was directly related to the amount of lipemic serum added (100 mL of lipemic serum reduced pool-two absorbance by 14%). Pool one, with a lower concentration of oligosaccharide-dye product, had a similar pattern of precipitate formation. However, a suspended precipitate, indicating a transition from a sinking to a floating precipitate, formed in the tube with only 25 mL of lipemic serum. The tubes with 10 and 50 mL of lipemic serum contained sinking and floating precipitates, respectively. These solutions were clear, but the absorbance was less than the initial absorbance of pool one. The solution in the tube containing 100 mL of lipemic serum remained slightly lactescent after floation of the precipitate by centrifugation.

When the final acid diluent was changed from sodium carbonate–sodium bicarbonate buffer (50 mmol/L, pH 10.0) no

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**Table 1. Effect of Serum Blank Procedure on the Amylochrome Assay in the Presence and Absence of Lipemia**

<table>
<thead>
<tr>
<th>Triglyceride, g/L</th>
<th>Test absorbance</th>
<th>Uncorrected acty, dye units/100 mL</th>
<th>Blank absorbance</th>
<th>Blank-corrected acty, dye units/100 mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>11.9</td>
<td>0.132</td>
<td>140</td>
<td>0.102</td>
<td>31</td>
</tr>
<tr>
<td>12.8</td>
<td>0.143</td>
<td>152</td>
<td>0.086</td>
<td>65</td>
</tr>
<tr>
<td>29.4</td>
<td>0.138</td>
<td>137</td>
<td>0.066</td>
<td>72</td>
</tr>
<tr>
<td>51.2</td>
<td>0.076</td>
<td>75</td>
<td>0.040</td>
<td>35</td>
</tr>
<tr>
<td>45.0</td>
<td>0.104</td>
<td>102</td>
<td>0.107</td>
<td>0</td>
</tr>
<tr>
<td>72.0</td>
<td>0.300</td>
<td>295</td>
<td>0.350</td>
<td>2</td>
</tr>
<tr>
<td>1.8</td>
<td>0.180</td>
<td>192</td>
<td>0.022</td>
<td>170</td>
</tr>
<tr>
<td>1.4</td>
<td>0.070</td>
<td>74</td>
<td>0.022</td>
<td>52</td>
</tr>
</tbody>
</table>

**Table 2. Measurement of Increased Amylose Activity by the Amylochrome and Maltotetraose Techniques in the Presence and Absence of Lipemia**

<table>
<thead>
<tr>
<th>Patient</th>
<th>Triglyceride, g/L</th>
<th>Amylose in urine, dye units/h</th>
<th>Lipemic sample, dye units/100 mL</th>
<th>Clarified sample, dye units/100 mL</th>
<th>Lipemic sample, maltotetraose substrate, U/L</th>
<th>Clarified sample, U/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>—</td>
<td>1528</td>
<td>211</td>
<td>357</td>
<td>210</td>
<td>204</td>
</tr>
<tr>
<td>2</td>
<td>57.1</td>
<td>18400</td>
<td>860</td>
<td>1320</td>
<td>914</td>
<td>923</td>
</tr>
<tr>
<td>Prepared sample</td>
<td>61.7</td>
<td>—</td>
<td>27</td>
<td>710</td>
<td>496</td>
<td>511</td>
</tr>
</tbody>
</table>

* a Clarified after overnight refrigeration. b Clarified by ultracentrifugation. c Blank absorbance exceeds test absorbance.

* Results are corrected for volume displacement based on apparent sodium concentration by flame photometry before and after clarification by ultracentrifugation: Patient 1, 7.8%; Patient 2, 5.5%, prepared sample, 8.7%.
Another major cause for underestimating serum amylase activity can be observed when above-normal amylase activity is present with lipemia. The blue precipitate, which forms only in the test but not in the blank, is induced by the production of a high concentration of the soluble oligosaccharide-triazinyl dye reaction product. This polyamionic product (10) at pH 4.3 appears to complex with lipoprotein components in the serum, and results, after routine centrifugation and removal of the blue precipitate, in partial or complete removal of lactescence only in the test sample. Analysis of the results of the serial mixtures of oligosaccharide–dye product and lipemic serum indicates that the amount of lactescence removed and the density of the precipitate formed depends on the ratio of the concentration of oligosaccharide–dye product and lipoprotein in the assayed sample. The loss of soluble oligosaccharide–dye product that is measured as reduced absorbance after the blue precipitate is removed along with any remaining lactescence due to lipemia is evidence that the reaction product is a component of the blue precipitate. Decreasing lactescence by precipitating lipoprotein with oligosaccharide–dye product results in an underestimation of amylase activity, because an appropriate correction for the remaining lactescence and for oligosaccharide–dye product loss cannot be generated by any manipulation of a serum blank procedure.

All lipemic samples that we have obtained from patients who have high serum amylase activity have produced floculent blue precipitates in the Amylochrome assay. The ability to form a precipitate is lost as the degree of lipemia is diminished, even though serum amylase activity remains above normal.

The lack of any effect by hypertriglyceridemia on the maltotetrose amylase assay suggests that hypertriglyceridemia has no actual effect on amylase activity but interferes with the Amylochrome assay due to its direct interaction with the soluble oligosaccharide–triazinyl dye reaction product. The possibility that other inhibitors, present only during hypertriglyceridemia, interfere with the measurement of amylase activity was not confirmed, because the amylase activity of the clarified serum samples from the two patients with hyperlipemia and acute pancreatitis remained proportionately unchanged when the samples were diluted.

We agree with the earlier brief observations by Gitlitz and Frings (8) and Ladenson et al. (7) that the Roche Amylochrome procedure is unsuited for measuring amylase activity in lipemic serum samples. We suggest that an alternative procedure such as the maltotetrose assay or sample clarification by ultracentrifugation be used for assessment of serum amylase activity in lipemic serum. The possibility that the Amylochrome procedure could be used for lipemic samples if the two-step blank and a final basic rather than acid diluent are used is now being investigated.

This work was supported in part by the Gundersen Medical Foundation. We thank Joe Tiedt for photography and Joyce Cartwright and Carol Boettcher for expert secretarial assistance.

References
4. Fallat, R. W., Vester, J. W., and Glueck, C. J., Suppression of
An Improved Assay of Mammalian Collagenase Activity, and Its Use to Determine Hepatic Extracellular Matrix Susceptibility to Degradation

William J. Lindblad and George C. Fuller

This rapid, sensitive method of determining collagenase (EC 3.4.24.7) activity incorporates several advantages of previous methods. Soluble [14C]acetylated collagen is prepared as the enzyme substrate and collagen-cleavage products are separated from noncleaved collagen by precipitation with dioxane/methanol. The assay is more reproducible than previous methods and has a lower detection limit, 15 mU of enzyme activity. We used the method in a competitive substrate assay with isolated extracellular hepatic matrix from cirrhotic and normal rat liver. Purified collagenase was consistently bound to normal rat matrix to a greater extent than to cirrhotic matrix, suggesting that in hepatic fibrosis the extracellular matrix is not as susceptible to collagenase degradation as that in normal liver.

Additional Keyphrases: cirrhosis • hepatic fibrosis • radioenzymic assay • rat liver tissue matrix

The maintenance of normal collagenous stroma depends on a dynamic equilibrium between collagen biosynthesis and degradation. The calcium-dependent neutral protease, collagenase (EC 3.4.24.7), is the principal extracellular enzyme responsible for collagen degradation in mammals. Since the initial reports of the enzyme in vertebrates (1), the enzyme has been localized in a wide variety of mammalian tissues and cells (2–5). Recently, several reports have implicated this enzyme in the pathological destruction of collagen in such disorders as rheumatoid arthritis (6) and recessive dystrophic epidermolysis bullosa (7). A role for this enzyme in tumor metastasis has also been proposed (8).

Previous studies have generally relied on a collagenase assay based on the cleavage of insoluble radiolabeled collagen gels in which the extent of radioactivity released is indicative of enzymatic cleavage. This method, first described by Gross and LaPierre (1), has several inherent disadvantages, including a long incubation (~18 h) and the inability to alter substrate/enzyme ratios. To circumvent these difficulties, a collagenase assay was needed in which soluble collagen was used as substrate. In the method we describe here, precipitation with an organic solvent is used to separate cleaved denatured collagen from native, triple-helical collagen. This approach, originally described by Terato et al. (9), was further refined by Ishikawa and Nimni (10). We have modified these procedures to provide a more nearly complete precipitation of native uncleaved substrate and to increase the sensitivity through use of a substrate with high specific activity, which is readily prepared in large quantities. These modifications allow measurement of enzyme activity in a 90-min incubation with good reproducibility and high sensitivity in detecting enzyme activity recovered from hepatic tissue.

We used this assay in several studies of hepatic collagen metabolism, including examination of the susceptibility of extracellular matrix to degradation by purified mammalian collagenase. These studies assessed the competition of extracted hepatic collagenous matrix and labeled substrate for binding to purified enzyme.

Materials and Methods

Reagents: All chemicals used in this study were of analytical grade or better, e.g., scintillation grade 1,4-dioxane (Malinckrodt Inc., St. Louis, MO 63147). Specific reagents include [1-14C]acetic anhydride (spec. acty. = 5.3 Ci/mol; New England Nuclear Corp., Boston, MA 02118) and Bray's solution (Research Products International Corp., Elk Grove Village, IL 60007).

Substrate preparation: Purified Type I calf-skin collagen was acetylated with [14C]acetic anhydride as described by Gisslow and McBride (11). Over a 2-h period, 0.5 mCi of [14C]acetic anhydride was added to 62.5 mL of a 2.0 g/L solution of collagen, with constant stirring at 10 °C (pH = 8.00). This solution was acidified with acetic acid to a pH of 4.00, then dialyzed for 96 h vs de-ionized water at 4 °C. The dialyzed substrate was diluted with an equal volume of 1 mol/L acetic acid, and 50 mg of peptic enzyme (EC 3.4.4.1) was added. This mixture was slowly stirred for 18 h at 4 °C, then dialyzed against de-ionized water for 48 h. The resulting substrate was...