Fluorescence Depolarization Assay for Quantifying α-Amylase in Serum and Urine

Marybeth Hofman and Mark Shaffar

We have developed a new method for quantifying α-amylase (EC 3.2.1.1) in serum and urine by fluorescence depolarization. Amylase in the sample catalyzes the hydrolysis of the substrate, a fluorescein-labeled amylase. This results in decreased fluorescence polarization, owing to the increased rate of rotation of the amylase fragment relative to the intact substrate. The TDx amylase assay is calibrated with six human-serum-based pancreatic amylase calibrators. Amylase activities are determined by interpolation from the calibration curve, which is stored in the TDx analyzer’s memory. Results correlate well with those by the Du Pont aca assay and the Beckman “DRI-STAT” assay. Endogenous glucose does not interfere. CVs are <5%, and the reagents are stable in liquid form.

Quantification of α-amylase (EC 3.2.1.1) in body fluids has long been important in diagnosing pancreatic disorders. Amylase activities in serum increase within a few hours of onset of acute pancreatitis and remain high for one to three days before returning to normal values (1). Prompt diagnosis is critical, to differentiate disorders that can be corrected surgically from acute pancreatitis, which presents surgical risks. Other disorders associated with increased amylase activities in serum include chronic pancreatitis, intra-abdominal disease, mumps, ectopic pregnancy, and alcoholism (2).

Techniques for quantifying amylase activity in serum include saccharogenic, amylolastic, chromogenic, and enzyme-coupled reactions (3–7). In saccharogenic assays and amylolastic assays, poorly defined substrates are used and the assays are time consuming. Assays in which defined substrates are used have been introduced in the past decade. In one, amylase hydrolyzes starch to maltose or dextrins, which are subsequently hydrolyzed by α-glucosidase to glucose (3, 4). When the rate of glucose production is used to estimate amylase activity, interference from endogenous glucose necessitates using a pre-incubation or pretreatment step. Another coupled-enzyme method is based on the rate of production of NADH from NAD" at 340 nm; it involves the use of starch, phosphorylase, β-phosphoglucomutase, and glucose-6-phosphate dehydrogenase (7). Although this method is free from endogenous glucose interference, several enzymes are required; moreover, the reagents are stable in liquid form for only one day at 2–8 °C.

The TDx® amylase assay is based on the principle of fluorescence polarization (8); the degree of polarization of light emitted from a fluorophore excited by a polarized beam of light is inversely related to how much the molecule containing the fluorophore has rotated between the time of excitation and emission. The rate of rotation, in turn, depends on the size or volume of the molecule.

In a fluorescence depolarization assay, a highly polarized fluorescein-labeled substrate is cleaved by the catalytic action of a macromolecular hydrolase, resulting in a decrease in the fluorescence polarization. In the TDx amylase assay, the fluorescein-labeled substrate is amylase, the natural substrate for α-amylase. When the 1,4-glucosidic linkages of the fluorescein-labeled amylase are hydrolyzed, the smaller fluorescent fragments formed are less polarized because they rotate faster than the intact substrate. In the TDx Analyzer, the polarization of the fluorescence in the reaction mixture is determined by sequentially exciting the reaction mixture, first with vertically and then with horizontally polarized light, and then analyzing only the vertical component of the emitted light.

The TDx fluorescence depolarization assay for quantification of α-amylase is free from interference from endogenous glucose and does not require the use of coupled enzymes. Because the reagent is stable in liquid form, reconstitution of reagents is not required. The simplicity of this direct and sensitive assay results in an assay that is free from other interferences such as are common to coupled-enzyme reaction systems.

Materials and Methods

Apparatus: We used the "TDx Fluorescence Polarization Analyzer" and the "VP Bichromatic Analyzer" (both from Abbott Laboratories, North Chicago, IL 60064), and the aca discrete analyzer (Du Pont Instruments, Wilmington, DE 19888).

Calibrators: The TDx amylase assay is calibrated with six human-serum-based calibrators that contain human pancreatic amylase, in activities ranging from 0 to 750 U/L. The activities of the calibrators were assigned so as to correlate with the aca amylase method (4). The calibration curve, derived from a four-parameter curve fit, is stored in the TDx Analyzer memory.

Reagents: TDx buffer (Abbott Laboratories) contained, per liter, 0.1 mol of sodium phosphate, 0.1 g of bovine gamma globulin, and 1 g of sodium azide. Beckman "Amylase D.S." reagents were obtained from Beckman Instruments Inc., Carlsbad, CA 92008. aca amylase reagent packs and "Enzyme Diluent" were obtained from Du Pont Instruments. Amylase and fluorescein isothiocyanate were from Sigma Chemical Co., St. Louis, MO 63178; dimethyl sulfoxide (DMSO) from Aldrich Chemical Co., Milwaukee, WI 53233; dibutyltin dilaurate from Alfa Products, Thiokol/Ventron Division, Danvers, MA 01923; and salivary and pancreatic amylase standards from Pharmacia Diagnostics, Piscataway, NJ 08854.

TDx reagent preparation: We prepared fluorescein-labeled amylase by a method similar to that of De Belder and Granath (10). We dissolved 10 g of amylase in 200 mL of DMSO, then added two drops of pyridine, 14 mL of dibutyltin dilaurate, and 10 mg of fluorescein isothiocyanate and heated the solution to about 100 °C for 1 h. After it cooled, we purified the labeled substrate by adding 800 mL of ethanol and cooling to −20 °C to precipitate the amylase. The precipitated amylase was filtered and redissolved in 200 mL of DMSO, then re-precipitated with ethanol. We repeated the precipitation and filtering procedures two more times.
to remove all free fluorescein. Finally, we dissolved the precipitated amylase in 200 mL of DMSO and used this, after adding NaCl to a final concentration of 15 mmol/L, as the stock for the reagents for the TDx amylase assay (prepared by further dilution with DMSO). The reagent is stable for at least four months when stored at 2–8 °C.

**TDx assay procedure:** The TDx reagents are stored in liquid form in a bar-code-labeled unit-dose cartridge with five wells. Three wells contain liquid reagent and stay sealed until used; two of these wells contain serum pretreatment solutions, the third contains the fluorescein-labeled amylase. The fourth and fifth wells are empty: one is for the patient's sample, the other for use by the TDx Analyzer in its pipetting sequence.

During the first revolution, the TDx Analyzer dispenses into the cuvette 12.5 μL of the pretreatment solution, 12.5 μL of sample, and 975 μL of TDx buffer. After the blank is read, 37.5 μL of pretreatment solution, 25 μL of the fluorescein-labeled substrate, 12.5 μL of the sample, and additional TDx buffer are added, to a final reaction volume of 2 mL. After a 6-min incubation the final polarization is measured. To correct for the background fluorescence of the sample, the Analyzer subtracts the blank reading from the final reaction mixture reading before calculating the change in polarization. The standard curve is generated with the corrected polarization values and amylase activity is determined by interpolation from the stored standard curve.

**Results and Discussion**

**Calibrators:** Figure 1 shows a typical calibration curve and illustrates the changes in calibration curves prepared with reagents stored at 45 °C and 2–8 °C for 60 days.

The calibration curve is stable for at least two weeks. Controls at three activity concentrations were assayed in duplicate over a period of 14 days and their concentrations determined by comparison with a single standard curve run on the first day of the study. Results for the low control (expected range 60–73 U/L) were 66.8, 71.4, and 63.5 U/L on days 1, 8, and 14, respectively; for the median control (expected range 160–196 U/L), results were 196.4, 189, and 174 U/L. The higher control (expected range 410–460 U/L) measured 430, 453, and 415 U/L on the same respective days.

The calibrators are stable for 10 days at 37 °C and 45 °C, and for at least four months at 2–8 °C. Table 1 summarizes the results of assaying the calibrators over a four-month period by the Beckman D.S. Amylase method (7).

**Dynamic range:** The TDx amylase assay is calibrated between 0 and 750 U/L, a range that includes the activities encountered in most patients' samples. Amylase activities in healthy individuals range from about 20 to 90 U/L; thus the highest calibrator is about eight times the upper limit of normal. When amylase activities in samples exceed the limit of the highest calibrator, the instruments prints out "HI". Such samples must be diluted in TDx buffer or saline and re-assayed.

**Precision:** Between- and within-run precision were determined by assaying three activity concentrations of human pancreatic amylase controls—three carousels of 20 replicates of each concentration—with the TDx Analyzer on the same day. The between- and within-run CVs for the low control (mean, 81.5 U/L) were 4.5 and 5.5%, respectively; 2.0 and 2.1% for the medium control (213 U/L); and 4.4 and 4.8% for the high control (452.4 U/L).

**Isoenzyme specificity:** Two controls, one containing pure salivary amylase and one containing pure pancreatic amylase, were assayed with the TDx Analyzer and the aca. The pancreatic control results were 79 U/L on the TDx and 64 U/L on the aca, whereas the salivary control results were 166 and 181 U/L, respectively. Both isoenzymes catalyze the hydrolysis of substrate in the TDx and aca assays, although the rate appears to be slightly faster for the pancreatic isoenzyme in the TDx assay.

**Interferences:** We also prepared controls by adding human pancreatic amylase to pooled human serum, then added potential interferents to aliquots of the controls. Hemoglobin interference was determined by adding hemolysate to the controls. Triglyceride interference was determined by adding human pancreatic amylase into a sample containing high concentration of triglyceride and determining the percent recovery of the added amylase. Ethanol (4.0 g/L), uric acid (200 mg/L), urea (2.4 g/L), creatinine (350 mg/L), glucose (10.0 g/L), fructose (10.0 g/L), ascorbate (250 mg/L), hemoglobin (1.50 g/L), glutathione (500 mg/L), and sodium heparin (143 USP units/L) do not interfere with the assay. Bilirubin at 150 mg/L decreases apparent amylase activity by 12%. Hemolysis and lipemia do not interfere with the assay. We recovered 100% of the added amylase from a sample containing 8 g of triglycerides per liter.

**Specimen collection:** Serum and heparin-anticoagulated specimens are satisfactory. Specimens containing EDTA, oxalate, fluoride, or citrate—which are amylase inhibitors—should not be used (11).

**Table 1. Stability of Six Amylase Calibrators**

<table>
<thead>
<tr>
<th>Day 1</th>
<th>Day 10</th>
<th>Day 70</th>
<th>Day 125</th>
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<tr>
<td>2-8 °C</td>
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<td>37 °C</td>
<td>45 °C</td>
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<td>182.9</td>
<td>189.9</td>
<td>193.7</td>
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<tr>
<td>594.7</td>
<td>585.8</td>
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<tr>
<td>964.0</td>
<td>937.8</td>
<td>964.4</td>
<td>1000.0</td>
</tr>
</tbody>
</table>

Fig. 1. Typical calibration curves for TDx amylase assay, prepared with reagents stored at 2–8 °C (C) and 45 °C (Θ) for 60 days. Ordinate, arbitrary polarization units.
Table 2. Correlation of Results for Patients' Sera: TDx vs aca vs Beckman

<table>
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<tr>
<th>Comparison</th>
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<th>r</th>
<th>S_yx</th>
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<tr>
<td>TDx vs aca</td>
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<td>aca vs Beckman</td>
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<td>42</td>
<td>0.97</td>
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</table>

Method correlation: Serum samples collected from patients were stored at 2–8 °C until assay by the aca amylose assay (4), the Beckman D.S. amylose assay (7), and the Abbott TDx amylose assay. We ran the Beckman D.S. assay in the Abbott VP according to the reagent manufacturer’s instructions. The correlation of results is summarized in Table 2.

Urinary amylose: We assayed singly nine urine samples ranging from 75 to 1200 U/L, using both the TDx analyzer and the aca. For the latter, all urine samples were diluted with protein-containing diluent according to the manufacturer’s instructions. For the TDx determination, one sample had to be diluted with TDx buffer to fall within the calibrator range. The linear regression equation for the results by the TDx (y) vs those by the aca (x) is y = 4.4 + 0.96x (r = 0.99, S_yx = 13.4).

References