Kinetic Determination of Serum Sorbitol Dehydrogenase Activity with a Centrifugal Analyzer

Joseph F. Dooley, 1 Lorraine J. Turnquist, and Linda Racich

We describe a mechanized method for centrifugal analyzer determination of sorbitol dehydrogenase in serum, based on conversion of D-fructose to sorbitol with simultaneous oxidation of NADH, in triethanolamine buffer at pH 7.4 and 30 °C. The standard curve for this assay is linear to 200 U of activity per liter of serum. The mean within-run precision (CV) of the assay is 0.8%. Results correlate well with those by a spectrophotometric method. In sera from 20 apparently healthy adult humans, sorbitol dehydrogenase activity averaged 1.7 (SD, ±0.8; range, 1–3) U/L. The mean activity (U/L) for a group of 30 rats was 4.4 (SD, ±0.2; range, 3–6); for 20 dogs, 5.8 (SD, ±0.7; range 3–9); and for 30 mice, 26.8 (SD, ±2.1; range, 22–34). To determine the utility of measuring this enzyme in the serum of rats for assessment of hepatotoxicity in drug-safety studies, we compared sorbitol dehydrogenase activity with that of alkaline phosphatase, aspartate aminotransferase, and alanine aminotransferase in the sera of rats treated with thioacetamide or in which the common bile duct had been ligated.

Additional Keyphrases: enzyme activity • normal values (human, dog, rat, mouse) • assessing drug hepatotoxicity • veterinary clinical chemistry • activity in serum and erythrocytes

A more easily used laboratory procedure is needed for detection of hepatotoxicity, to be used in the assessment of safety of chemicals and drugs. This method should be both specific and sensitive, and should also be clinically useful.

Sorbitol dehydrogenase (SDH; L-iditol:NAD+ 2-oxidoreductase, EC 1.1.1.14) has been identified in several human and animal tissues (1–5). It is located primarily in the cytoplasm and mitochondria of the liver, kidney, and seminal vesicles (6, 7). The use of the SDH assay is based on the finding that SDH activity in the serum is normally low but increases during acute episodes of liver damage (8, 9). Measurement of SDH activity is therefore useful as a specific indicator of liver-cell damage (10, 11).

Assay of SDH involves oxidation of NADH at 340 nm, coupled to the conversion of D-fructose to sorbitol at 30 °C (9). We describe the assay conditions for SDH assay with a centrifugal analyzer, and discuss its usefulness for detection of hepatotoxicity and its sensitivity in comparison with better-known enzyme assays used for this purpose—alkaline phosphatase (EC 3.1.3.1), aspartate aminotransferase (EC 2.6.1.1), and alanine aminotransferase (EC 2.6.1.2)—as studied in rats in which hepatotoxicity was induced with thioacetamide and by bile-duct ligation.

Materials and Methods

Apparatus

We used a Centrifichem series 400 centrifugal analyzer and pipettor (Union Carbide Corp., Rye, NY 10580). Spectrophotometric determinations were made with a Model 2400S spectrophotometer (Gilford Instrument Laboratories, Oberlin, OH 44074) equipped with a Lauda Model K 2/R temperature regulator (Brinkmann Instruments, Westbury, NY 11590).

Reagents

Triethanolamine buffer: Dissolve 37.2 g of triethanolamine hydrochloride in distilled water, adjust the pH to 7.4 with sodium hydroxide (2.0 mol/L), and dilute to 100 mL with distilled water.

β-NADH, 6 mmol/L: Dissolve 15 mg of β-NADH (cat. no. 340–1100; Sigma Chemical Co., St. Louis, MO 63178) in 3.0 mL of NaHCO₃ solution (10 g/L). This solution is stable for one week at 4 °C.

D-Fructose, 4 mol/L: Dissolve 72.1 g of D-fructose in 100 mL of distilled water. This solution is stable for one month at 4 °C.

Lactate dehydrogenase (EC 1.1.1.27): 5.5 U/L (cat. no. 127868; Biodynamics/bmc, Indianapolis, IN 46250).

Procedures

Assay: To prepare the substrate solution, add 2.4 mL of fructose solution, 0.6 mL of NADH solution, and 1.0 mL of lactate dehydrogenase solution to 9.6 mL of triethanolamine buffer. With a Centrifichem pipettor, add 25 μL of sample and 50 μL of water (sample + diluent) to the sample well, and 250 μL of substrate solution to the reagent cavity. Program the centrifugal analyzer as follows: temperature = 30 °C, t₀ = 30 s, Δt (time interval of subsequent readings) = 60 s, number of prints = 3, autoblock, rate mode, concentration factor = 1690.

Other assays: Certain sera that were tested for SDH activity by the present method were also assayed for alkaline phosphatase activity by the method of Wilkinson and Boutwell (12), 5'-nucleotidase (EC 3.1.3.5) activity by the method of Sunderman et al. (13), alanine aminotransferase activity by the method of Wroblewski and LaDue (14), and aspartate aminotransferase activity by the method of Karmen (15).

Introduction of biliary obstruction in rats: We induced acute biliary obstruction in male Sprague–Dawley rats (mean body weight, 250 g; range, 200–300 g) by bile-duct ligation as described by Issa et al. (16) and Kryszewski et al. (17). Control rats were anesthetized with pentobarbitol and their abdominal cavities were opened by midline incision. The bile duct was sham-ligated by passing a ligature loosely around the common bile duct in each control rat; the ligature was removed before

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the abdominal incision was closed. Blood was obtained from the test rats and control rats from the vena cava two, three, and four days after the operation.

Induction of hepatotoxicity in rats: We induced acute toxic injury of hepatic parenchymal cells in male Sprague–Dawley rats (same characteristics as above) by oral administration of thioacetamide, 25 or 50 mg/kg of body weight, dissolved in 2.5 mL of water, as described by Korsrud et al. (18). Control rats received 2.5 mL of water postoperatively, by gavage. After the gavage, the rats were fasted, but were permitted free access to water. We obtained blood from the vena cava of each test rat and control rat 24 h after the gavage.

Results

Optimum Concentration of Fructose

To assess the optimum concentration of D-fructose needed for maximum SDH activity, we used pooled sera from 10 male rats with chemically induced hepatic necrosis, and another pool of sera from 10 normal male rats. As shown in Figure 1, SDH activity in normal rat serum is maximum for concentrations of fructose between 2.5 and 3.5 mol/L. In sera of rats containing very high SDH activity, enzyme activity is maximum at about 3.0 mol of fructose per liter. The SDH activity of pooled human and beagle dog sera as a function of the concentration of fructose solution is also shown in Figure 1. There is a 9% increase in activity from 2.0 to 4.0 mol/L of fructose per liter for human serum, with no further increase above 4.0 mol/L. Dog serum shows a more substantial (44%) change in SDH activity with increasing fructose concentrations, but again no further increase is seen above 4.0 mol/L.

Assay Conditions with the CentriFichem

We monitored, at 30 °C, the change in absorbance after mixing the sample and diluent with the substrate solution as described in the Procedures. The concentrations of the components of the enzymatic reaction mixture were: per liter: D-fructose, 3.1 mol; NADH, 4.6 mmol; buffer, 27.3 mmol; and serum, 76.9 mL. The linearity of the plot of change in absorbance vs time from 1.5 to 6.5 min was confirmed with use of Caltrol II (Calbiochem control serum; supplier-assayed SDH value = 57 U/L). The interval of printing absorbance measurements was 30 s.

Comparison with Analysis by a Manual Method

We analyzed serum samples from rats, mice, dogs, and humans for SDH activity with the present method and with a manual spectrophotometric method, performed according to the method of Gerlach and Hiby (9). The results are plotted in Figure 2. Measurements with the centrifugal analyzer (y) were comparable to those of the manual method (x) (p < 0.10) (19). The correlation coefficient was 0.90.

Analytical Precision

Within-run precision of our method was estimated from 10 consecutive assays of Caltrol reference serum. The mean (±SD) SDH activity was 61.1 U/L (±0.54), and the within-run CV was 0.8%.

Day-to-day precision of the automated method was measured on the basis of analyses of a single pooled mouse serum on 15 consecutive working days. 100-μL aliquots of pooled serum were frozen in 15 tubes and stored at −20 °C. One tube was thawed for each day’s analysis. The mean (±SD) SDH activity of the pooled sera was 31.0 U/L (±3.0), and the day-to-day CV was 9.7%.

Effect of Hemolysis

To test the effect of erythrocyte hemolysis on the assay of serum SDH activity, we divided samples of dog and human whole blood into two 1-mL portions. One portion was hemolyzed by forcing the blood through a 23-gauge needle. Both samples were allowed to clot at room temperature, and non-hemolysed and grossly hemolysed (serum hemoglobin concentration, 0.6 g/L) specimens were analyzed for serum SDH activity. Analysis of eight paired dog sera by the automated method showed a 54% mean increase in SDH activity after hemolysis. The activity in seven paired human sera increased 30% after hemolysis.

Stability of Serum SDH

To measure the stability of serum SDH activity, we divided a pooled rat serum sample into several 100-μL aliquots. Four aliquots were allowed to remain at 25 °C for up to 4 h; six samples were stored at 4 °C for up to two weeks; and four tubes were stored frozen for one to four weeks. At 1 and 4 h (for the samples at 25 and 4 °C), and at one-, two-, three-, and four-week intervals, we analyzed the serum aliquots for SDH activity. It remained stable for up to 4 h at 25 °C; at 4 °C, it decreased by 6.5% after one week and by 12.6% after two weeks of storage. Storage at 0 °C resulted in essentially no change (5.0%) after one week, a decrease of 8.6% after two weeks, and a 13.7% decrease after three and four weeks.

Normal Values

We obtained normal (expected) values for serum SDH by
Table 1. Effects of Acute Hepatotoxicity Induced by Thioacetamide on Enzyme Activities in Rat Sera

<table>
<thead>
<tr>
<th>Thioacetamide, mg/kg body wt</th>
<th>No. of rats</th>
<th>ALT, U/L</th>
<th>AST, U/L</th>
<th>ALP, U/L</th>
<th>SDH, U/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>51</td>
<td>20</td>
<td>19 ± 3</td>
<td>61 ± 7</td>
<td>193 ± 17</td>
<td>7 ± 2</td>
</tr>
<tr>
<td>20</td>
<td>20</td>
<td>180±12</td>
<td>1675±25</td>
<td>251±43</td>
<td>72±5</td>
</tr>
</tbody>
</table>

ALT, alanine aminotransferase; AST, aspartate aminotransferase; ALP, alkaline phosphatase. *p < .0001.

(comparing the serum activities of 20 humans, 20 dogs, 30 rats, and 30 mice. The serum samples from humans were from apparently healthy volunteers at Pfizer Central Research. Serum from dogs was from blood drawn from the jugular vein of healthy, one-year-old Marshall Farms male beagles. Serum was also obtained from the blood of Charles River Laboratories male albino rats (body weight, 200 g) and mice (15 g), after sodium pentobarbital anesthesia and exsanguination from the vena cava. The mean SDH activity was 1.7 (SD, ±0.8) U/L (range, 1–3 U/L) in human sera; 5.8 (±0.7) U/L (range, 3–9) in dogs; 4.4 (±0.2) U/L (range, 3–6) in rats; and 26.8 (±2.1) U/L (range, 22–34) in mice. The reference interval (5th to 95th percentiles) for serum SDH activity was calculated by the nonparametric method of Herrera (20).

Detection of Hepatotoxicity

We measured the activities of alanine aminotransferase, aspartate aminotransferase, alkaline phosphatase, and SDH in sera from rats administered 25 mg of thioacetamide per kilogram of body weight (Table 1). In sera obtained from rats 24 h after this oral administration, the mean activities of serum enzymes in the dosed group increased by 9.5- and 11.1-fold for alanine aminotransferase and aspartate aminotransferase, and 10.3-fold for SDH. Alkaline phosphatase activity did not change significantly within 24 h.

To examine the sensitivity of serum SDH activity to acute hepatic necrosis, we gave 20 male rats 0, 5, 7, 10, 15, 25, and 50 mg of thioacetamide per kilogram of body weight, intra-peritoneally, and measured the activity of the same four enzymes 24 h later. Table 2 summarizes the results of this experiment. All enzyme activities measured differed significantly from those in control rats dosed with thioacetamide at 50 and 25 mg/kg. At the 15 mg/kg dosage, aspartate aminotransferase and SDH activities were substantially increased over controls; at 10 mg/kg, only SDH showed a statistically significant change. No significant changes in activities were evident at 7 or 5 mg of thioacetamide per kilogram.

We also performed experiments to determine whether SDH activity is increased in acute biliary obstruction. Five rats had their bile ducts ligated and were given free access to food and water after the operation. Five control rats had sham ligation of the bile duct. Blood was drawn for enzymatic analysis after three days. Results of measurements of alkaline phosphatase, 5'-nucleotidase, and SDH are summarized in Table 3. Serum activities of all these enzymes increased substantially after the ligation as compared with activities in the sham-operated rats.

During toxicity experiments, a beagle dog receiving high doses of an experimental compound was observed to have the following serum enzyme activities (U/L): SDH 38, alanine aminotransferase 620, aspartate aminotransferase 385, and 5'-nucleotidase 8. The respective expected ranges for the last three are 7–24, 17–43, and 1–8 U/L. Subsequent histopathological examination revealed severe hepatic centrolobular necrosis.

Discussion

The assessment of liver toxicity plays a central role in chemical- and drug-safety studies (21). When laboratory animals are used for toxicity studies, a wide variety of laboratory measurements are available for quantitation and comparison with histology. However, an easily measurable serum assay is needed that can be used effectively in test animals and can be compared directly in man. Asada and Galambo, in a classical paper (21), demonstrated that SDH can be used effectively as an index of hepatocellular damage in both animals and man. They pointed out that not only is SDH virtually specific for liver, but it also has the same organ distribution in man and rat. This is in distinct contrast to aspartate aminotransferase, which is distributed widely among many organs (e.g., heart, liver, kidney, and skeletal muscle) and differs substantially between rat and man in its relative activity in various organs. The presence in muscle tissue of the other popularly assayed enzymes of the glycolytic pathway such as lactate dehydrogenase preclude their usefulness in experimental animals for detection of drug-induced liver toxicity because of the use of repeated intramuscular injections, and the necessity for restraint during blood sampling. This report demonstrates that SDH activity is low in normal rat, dog, and human serum.

The method for SDH assay used here is based on the enzymic oxidation of NADH (22). The method was adapted for use with an automated system and with only 25 μL of serum. Previous methods require larger quantities of serum (23), and so are less useful in work with experimental animals.

Previous investigators (24) have reported no effect of hemolysis on serum SDH activity. We have found, however, that both dog and human erythrocytes contain enough SDH activity to alter serum SDH activity substantially if hemolysis is present. Rat serum SDH activity is stable (13.7% decrease).

Table 2. Mean Enzyme Activities in Serum from Rats Dosed with Thioacetamide, 24 h after Dosing

<table>
<thead>
<tr>
<th>Thioacetamide, mg/kg body wt</th>
<th>No. rats</th>
<th>ALT, U/L</th>
<th>AST, U/L</th>
<th>ALP, U/L</th>
<th>SDH, U/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>50</td>
<td>20</td>
<td>407*</td>
<td>732*</td>
<td>275*</td>
<td>350*</td>
</tr>
<tr>
<td>20</td>
<td>20</td>
<td>98b</td>
<td>203*</td>
<td>93*</td>
<td>114*</td>
</tr>
<tr>
<td>15</td>
<td>20</td>
<td>34</td>
<td>64c</td>
<td>56</td>
<td>19*</td>
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<td>28</td>
<td>45</td>
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<tr>
<td>0</td>
<td>20</td>
<td>25</td>
<td>45</td>
<td>63</td>
<td>6</td>
</tr>
</tbody>
</table>

*a p < 0.001. b p < 0.01. c p < 0.05. See Table 1 for abbreviations.

Table 3. Effects of Acute Biliary Obstruction on Enzyme Activities in Rat Sera

<table>
<thead>
<tr>
<th>Treatment</th>
<th>No. rats</th>
<th>Serum enzyme activities, U/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ligation</td>
<td>5</td>
<td>198b±59</td>
</tr>
<tr>
<td>Sham (controls)</td>
<td>5</td>
<td>84±12</td>
</tr>
</tbody>
</table>

 SDH 110±2

*a 5/N, 5'-nucleotidase. b p < 0.001.
for up to four weeks during storage at 0 °C, in substantial agreement with the previous report of Schmidt et al. (25), who observed no decrease in activity for 48 h at −18 °C.

Thioacetamide-induced centrolobular necrosis will increase SDH activity in the serum of rats to values easily distinguishable from those for controls. We conclude that the low SDH activity in normal serum and the rather substantial increase in the presence of liver-cell injury make SDH useful in identifying hepatotoxicity, and in assigning “no-effect” values as well. The response of serum SDH to toxic chemicals has been confirmed by others (26). The response of SDH to bile-duct ligation agrees with the findings of Moritz and Snodgrass (27), who reported a rapid rise of SDH after obstruction of the common bile duct in rats. The presence of extensive hepatocyte necrosis in bile-duct obstruction releases SDH from the cytoplasm. These conclusions are also reported in other experiments (17), where SDH activity was found to be associated with bile-duct ligation.

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References