Improved Assay for Alcohol Dehydrogenase Activity in Serum by Centrifugal Analysis

Shinzo Kato, Hiromasa Ishii, Shojiro Kano, Sachiko Hagihara, Toru Todoroki, Shigeyuki Nagata, Hisao Takahashi, Mari Nagasaka, Jun Sato, and Masaharu Tsuchiya

We describe an improved method for determination of alcohol dehydrogenase (EC 1.1.1.1) activity in 60 μL of human serum, based on conversion of ethanol to acetaldehyde with simultaneous reduction of NAD⁺ in glycine NaOH buffer (pH 9.0) at 37 °C in a centrifugal analyzer. The final concentration of NAD⁺ was 10 mmol/L and ethanol was 20 mmol/L. The dilution curve was linear with enzyme activity up to 200 U/L, and results by this method correlated well with those by a manual method (N Engl J Med 279: 241–248, 1968). Within-run precision (CV) was 0.9% over the range of 4.5 to 88.1 U/L, and day-to-day precision was 5.4% to 5.6%. In sera from 198 healthy individuals, mean alcohol dehydrogenase activity was 1.6 (SD 1.2, range 0–5) U/L. To evaluate the clinical utility of determining alcohol dehydrogenase, we measured the activity of alanine aminotransferase and alcohol dehydrogenase in sera from 470 patients with various diseases in our hospital, and found that results for the two enzymes did not correlate well.

Additional Keyphrases: liver disorders · enzyme activity · reference interval · alanine aminotransferase · hypoxia · shock · isoenzymes

Alcohol dehydrogenase (ADH; alcohol:NAD⁺ oxidoreductase, EC 1.1.1.1) is well known as a relatively liver-specific enzyme localized in the cytoplasm (I, 2), and has previously been tested for use as a good index of hepatic injury. But the clinical use of its determination in serum has been limited, its activity in normal human serum being too low to measure spectrophotometrically (3). The development of sensitive assays of serum ADH activity by Skursky et al. (4) and Buehler and Von Warburg (5) has led to recent reevaluation of the clinical significance of serum ADH. However, these methods are not well suited for use with large numbers of daily samples in the clinical laboratory, because they involve use of p-nitrosodimethylamine, a toxic (possibly carcinogenic) agent (4), or anti-ADH antibody, which is difficult to obtain (5). Therefore we have improved the assay method by modifying the procedure of Bonnichsen and Brink (6) and applying it to the centrifugal analyzer, which is simple, accurate, and sensitive. We then compared ADH activity in sera from patients with liver disease with their activity of serum alanine aminotransferase (ALT; EC 2.6.1.2).

Materials and Methods

Apparatus. For automatic analysis we used a centrifugal analyzer (Cobas Bio; La Roche, Basel, Switzerland). Manual spectrophotometric determinations were made with a Model 320 spectrophotometer (Hitachi Ltd., Tokyo, Japan).

Reagents. Glycine-NaOH buffer: Dissolve 7.507 g of glycine in distilled water, adjust to pH 9.0 with NaOH at 37 °C, and dilute to 1 L with distilled water. This solution is stable for one month at 4 °C.

NAD⁺ solution: Dissolve 531 mg of NAD⁺ (Boehringer Mannheim; cat. no. 127973) in 10 mL of distilled water. This solution was prepared freshly on each day of the assay.

Ethanol solution: Dissolve 1.85 g of ethanol in 50 mL of distilled water. This solution is stable for one month at 4 °C.

Procedure. To prepare substrate solution, add 2 mL of NAD⁺ solution to 10 mL of 0.1 mol/L glycine-NaOH buffer (pH 9.0). Add 30 μL of serum and 60 μL of distilled water (sample + diluent) to each sample well of the centrifugal analyzer, and 300 μL of buffered NAD⁺ solution to the reagent cavity. After 5 min of preincubation, start the enzyme reaction by adding 10 μL of ethanol solution or distilled water. Program the centrifugal analyzer as follows: type of analysis = 3, temperature = 37 °C, wavelength = 340 nm, incubation time = 300 s, time of first reading = 20 s, no. of readings = 18, mode of print = 1, concentration factor = 1323. The final concentrations of NAD⁺ and ethanol in the reaction mixture are 10 and 20 mmol/L, respectively. Certain sera tested for ADH activity by the present method were also assayed for ALT activity by the method of Karmen et al. (7).

Samples. Sera from patients with various disorders were obtained from our university hospital; these were brought to the laboratory as emergency samples for 10 consecutive days. Sera for determination of normal ADH activities were obtained from 198 apparently healthy individuals (ages 29–64 years, 71 men and 127 women), hospital employees who had had a medical examination. Serum was prepared from venous blood, stored at 4 °C, and analyzed within 30 h.

Results

When serum was mixed with the buffer containing NAD⁺, we observed the nonspecific reduction of NAD⁺ due to "nothing" dehydrogenase (8). This blank reaction was fast, being essentially linear in 5 min; thus we decided to preincubate for 5 min before adding ethanol to start the ADH reaction. For the reference blank, we added distilled water instead of ethanol. Subsequently we measured the change of absorbance (ΔA) at 340 nm, and used the difference of ΔA between sample and reference to calculate ADH activity as follows:

\[
\text{ADH activity, U/L} = \frac{\Delta A_{\text{sample}}/\text{min} - \Delta A_{\text{reference}}/\text{min}}{6.3 \times 10^3 \times 1.6} \times \frac{400}{30} \times 10^6
\]

\[
= (\Delta A_{\text{sample}}/\text{min} - \Delta A_{\text{reference}}/\text{min}) \times 1323
\]

Received June 27, 1984; accepted August 13, 1984.
We optimized various conditions of the reaction as listed below to obtain maximum activity of serum ADH. For these studies we used sera with high ADH activity.

**Buffer concentration and optimum pH.** We used 0.1 mol/L glycine NaOH buffer as previously described (6), varying the pH from 8.6 to 9.8. Optimum pH of the buffer was about 9.0 (Figure 1).

**NAD⁺ concentration.** We examined reagent solutions with final concentrations of NAD⁺ ranging from 0 to 15 mmol/L (Figure 2). The apparent $K_m$ for NAD⁺ in this assay was 1.54 mmol/L, and activity was maximum when NAD⁺ was 10 mmol/L.

**Ethanol concentration.** We also prepared reagent solutions with final concentrations of ethanol from 0 to 100 mmol/L (Figure 2). The $K_m$ for ethanol in this assay was 1.43 mmol/L, and maximal activity was obtained at an ethanol concentration of 20 mmol/L.

**Temperature.** The temperature of the assay was varied from 25 °C to 37 °C. The higher the temperature, the greater the activity; therefore, we performed the assay at 37 °C.

**Linearity.** We measured ADH activity after diluting two serum samples (original ADH activity, 538 and 162 U/L) with distilled water. The dilution curve for the assay was linear up to 200 U/L.

**Comparison with analysis by a manual method.** We obtained 45 serum samples from patients with various pathological conditions and measured ADH activity by this method (γ) and by the manual spectrophotometric method (α) described by Mezey et al. (3). The results correlated well ($r = 0.9867$); the equation for the regression line was $y = 5.13x - 2.38$.

**Analytical precision.** Table 1 shows the results for 20 replicate ADH determinations on each of five samples. Within-run variation (CV) was dependent on the ADH activity of the sample and ranged from 0.9 to 8.2%. Day-to-day precision of this method was based on the analysis of a single pathological serum on 16 consecutive working days. Aliquots (100 μL) of the serum were frozen in 16 tubes and stored at −80 °C. One tube was thawed for each day's analysis. The mean (±SD) ADH activity of the stock serum was 37.3 (±2.0) U/L, and the day-to-day CV was 5.4%. In addition, we prepared a control serum by adding human liver homogenate to pooled serum, and measured ADH activity in this on 103 consecutive days; the mean (±SD) ADH activity was 79.1 (±4.5) U/L, and the CV was 5.7%.

**Stability of serum ADH.** To measure the stability of serum ADH activity, we divided a serum with high ADH activity into 100-μL aliquots; stored each sample at 4 °C, −25 °C, and −80 °C; and analyzed the serum aliquots for 12 days. At 4 °C, ADH activity decreased by 45% after three days, and by 82% after nine days of storage. Stored at −20 °C, ADH activity decreased by 34% after three days, and by 76% after nine days. However, when the serum was kept at −80 °C, ADH activity remained unchanged after 12 days (and even after two months). Twenty-four sera stored at 4 °C lost an average of only 3.3% of their ADH activity in 24 h.

**Reference interval.** Serum ADH activity in healthy individuals showed log normal distribution (Figure 3). The mean (±SD) ADH activity was 1.6 (±1.2) U/L, and the upper limit of normal (97.5 percentile) was estimated at 5 U/L. There was a small but significant sex-related difference in ADH activity ($p < 0.01$): in men, the mean (±SD) was 1.9 (±1.3) U/L, and 1.4 (±1.0) U/L in women.

**Table 1. Within-Run Precision of This Method**

<table>
<thead>
<tr>
<th>ADH activity, U/L</th>
<th>Mean</th>
<th>SD</th>
<th>CV, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>4.5</td>
<td>0.37</td>
<td>8.2</td>
<td></td>
</tr>
<tr>
<td>8.1</td>
<td>0.38</td>
<td>4.8</td>
<td></td>
</tr>
<tr>
<td>14.6</td>
<td>0.39</td>
<td>2.7</td>
<td></td>
</tr>
<tr>
<td>42.2</td>
<td>0.40</td>
<td>0.9</td>
<td></td>
</tr>
<tr>
<td>68.1</td>
<td>1.04</td>
<td>1.2</td>
<td></td>
</tr>
</tbody>
</table>

$n = 20$ each.
Correlation between the activity of serum ADH and ALT in patients. As Figure 4 shows, the correlation between ADH and ALT in 470 serum samples was low ($r = 0.365$). A majority of the patients with relatively high ADH activity (ADH/ALT > 0.3) were in shock, which suggest that these high values were probably associated with hepatic centrilobular damage (Table 2). By contrast, 29 patients with relatively low ADH activity (ADH/ALT < 0.035) were suffering from chronic hepatitis (13 cases), uncomplicated liver cirrhosis (two), hepatocellular carcinoma (two), acute viral hepatitis (one), biliary tract disease (one), and undetermined liver disorders.

Discussion

The proposed method is, to our knowledge, the first assay capable of determining serum ADH activity in healthy individuals by conventional spectrophotometry. We made three improvements to previous procedures for the determination of ADH in serum. Firstly, we incubated for 5 min, mixing NAD$^+$ and serum, before starting the ADH reaction. Secondly, we increased the concentration of NAD$^+$ to more than 20-fold that in the method of Bonnichsen and Brink (6). These two modifications were made in consideration of "nothing" dehydrogenase activity in serum. Third, we adapted the method to a centrifugal analyzer, which automatically performed the assay and recorded the small change of absorbance with high accuracy. This last change was essential for measuring low activities of ADH, as are found in healthy individuals. This method has some advantages over the previous methods because of its simplicity, accuracy, sensitivity, and requirement of small sample volume. Because this method can also be adapted to multiple-channel automated analyzers, we consider it suitable for routine use in clinical laboratories.

The optimum pH of the buffer differed from that reported by Mezey et al. (3), but agreed with that reported by Shimasue et al. (9). This discrepancy can be explained by the racial differences reported by Harada et al. (10) and Yin (11); for most Japanese the optimum pH for ADH is around 8.8, whereas for the ADH most Caucasians have, the optimum pH is around 10.5. We also observed a few serum samples in which ADH activity was higher at pH 10.6 than at pH 9.0. From this point of view, the pH of the buffer will need to be changed for determining ADH activity in Caucasians.

In healthy individuals, serum ADH activity showed log normal distribution, with higher activity in men than in women, as with many other enzymes such as amiotransferases or lactate dehydrogenase. However, this difference is so small, that it can be disregarded in clinical evaluation.

After much controversy (12, 13), ADH was clearly shown by immunohistochemical methods (14) to be distributed predominantly in the hepatic centrilobular area. Therefore it is tempting to suppose that serum ADH activity can be a sensitive indicator of hepatic centrilobular injury. Indeed, in this study, we found a marked increase of serum ADH activity in cases of shock or hypoxia; one patient with high ADH activity, who died of a rupture of dissecting aneurysm of the aorta, showed centrilobular necrosis of the liver in autopsy. In agreement with these findings, Mezey et al. (3) demonstrated increased serum ADH activity in halothane-induced hepatitis as well as in hypoxic hepatic injury, both of which are known to cause hepatic centrilobular necrosis. Furthermore, we have shown experimentally that hepatic centrilobular necrosis produced by bromobenzene or anoxia is associated with marked enhancement of ADH in serum (15).

Many reports on lactate dehydrogenase isoenzyme 6 (LDH 6) have been made recently (16–18), the appearance of LDH 6 in the serum having been mentioned as a poor prognostic sign in cardiorespiratory failure. We disclosed

<table>
<thead>
<tr>
<th>Age (yr)</th>
<th>ADH (U/L)</th>
<th>ADH/ALT ratio</th>
<th>Remarks</th>
</tr>
</thead>
<tbody>
<tr>
<td>80/M</td>
<td>288</td>
<td>147</td>
<td>1.96 Myocardial infarction.</td>
</tr>
<tr>
<td>54/M</td>
<td>87</td>
<td>84</td>
<td>1.04 Gastric cancer. Malignant</td>
</tr>
<tr>
<td>59/M</td>
<td>89</td>
<td>141</td>
<td>0.63 Hepatocellular carcinoma,</td>
</tr>
<tr>
<td>68/M</td>
<td>189</td>
<td>362</td>
<td>0.52 Cardiogenic shock.</td>
</tr>
<tr>
<td>71/F</td>
<td>500</td>
<td>1018</td>
<td>0.49 Acute respiratory failure</td>
</tr>
<tr>
<td>83/M</td>
<td>77</td>
<td>245</td>
<td>0.31 Rupture of a dissecting</td>
</tr>
<tr>
<td>62/M</td>
<td>178</td>
<td>784</td>
<td>0.23</td>
</tr>
</tbody>
</table>
that LDH 6 was in fact ADH itself, and the LDH 6 appeared on electrophoretograms when serum ADH activity was more than 200 U/L because of hepatic injury due to circulatory disturbance (19). We observed that in preshock or hypoxia, ADH was mildly increased and LDH 6 did not appear.

Thus, clinical and experimental data suggest that serum ADH activity is a useful index, with high sensitivity and specificity, in the early detection of hepatic centrilobular injury.

This work was partly supported by the grant (no. 59480212) of Ministry of Education of Japan.

References