Lipoamide Dehydrogenase in Serum: A Preliminary Report

John W. Pelley, Gwynne H. Little, Tracy C. Linn, and Frank F. Hall

Lipoamide dehydrogenase was identified in serum and the optimal conditions for its assay at 30 °C were defined. The pH optimum in tris(hydroxymethyl)aminomethane buffer is 7.8, and activity is inhibited if buffer concentration exceeds 100 mmol/liter. Saturating concentrations of the substrates NAD⁺ and lipoamide are 3 mmol/liter and 5 mmol/liter, respectively. Activity is decreased eightfold when lipoic acid is substituted for lipoamide. Activity is linearly related to enzyme concentration up to a limiting absorbance change of 0.300 at 340 nm, and both within-day and day-to-day precision are satisfactory. Data suggest a normal range (2 SD) of 3–19 kU/liter. The highest value measured in serum was 473 kU/liter. A correlation with direct bilirubin concentrations (r = 0.435, P < 0.01) was found.

Additional Keyphrases: reference values • correlation with bilirubin

We report here for the first time the measurement in serum of activity attributable to lipoamide dehydrogenase (LAD),¹ which catalyzes the following reversible reaction:

\[
\text{lipoamide (red.) + NAD}^+ \leftrightarrow \text{lipoamide (oxid.) + NADH}
\]

In this initial report we document the presence of LAD in blood serum, and define the requirements and optimum conditions for its clinical assay. In addition, we have attempted to correlate above-normal LAD activity in serum with some established chemical indices of disease; the only positive correlation found was with values for direct bilirubin in serum.

Materials and Methods

Reagents

**LAD I (Tris–NAD⁺).** Add 432 mg of NAD⁺ (N-7004; Sigma Chemical Co., St. Louis, Mo. 63178) to 100 ml of 0.1 mol/liter Tris HCl, pH 7.8, and 40 ml of water. Readjust the pH to 7.8 and dilute to a final volume of 150 ml with water. Divide the solution into 10-ml aliquots and store at −20 °C until used. Thawed solutions are stable for at least three 8-h workdays at 30 °C with overnight storage at −20 °C.

**LAD II (Tris–DDT–Lipoamide).** Dissolve 40 mg of lipoamide (Sigma) and 60 mg of DTT in 2.0 ml of 95% ethanol. Add 6.0 ml of 50 mmol/liter Tris HCl, pH 8.2, and incubate for 30 min at 30 °C. Divide the solution into aliquots sufficient for one day's use, and store them at −20 °C. This solution is stable for 8 h at 30 °C; leftover amounts can be frozen and reused once without adverse effects.

Procedure

Assays were routinely performed with a Model 24 recording spectrophotometer (Beckman Instruments, Inc., Fullerton, Calif. 92634) with a constant-temperature cell holder equilibrated at 30 °C. Reagents LAD I and LAD II were pre-equilibrated at 30 °C and maintained at this temperature in a constant-temperature apparatus. The assays were done in a 1.4-ml cuvette with a 1.0-cm lightpath, as follows:

1. Add 750 μl of LAD I reagent.
2. Add 50 μl of serum or an appropriate dilution.
3. Preincubate for 10 min at 30 °C. Monitor nonspecific background with the spectrophotometer during this step.
4. Add 200 μl of LAD II reagent.
5. Record the increase in absorbance (A) at 340 nm. Calculate ΔA/10 min, subtracting background activity determined in step 3, as necessary.

The concentration (in mmol/liter) for each component in the 1.0-ml reaction mixture is: Tris HCl (pH 7.8), 0.057; NAD⁺, 3; lipoamide, 5; and DTT, 10. LAD values are reported in international (IUB) units per liter (U/liter) when assayed under the conditions described above. The calculation for a 50-μl sample is as follows:

\[
\text{U/liter} = \frac{(\Delta A_{340}/10 \text{ min}) \times (322 \times 10^3)}{\text{sample volume}}
\]

The factor 322 × 10³ takes into account the volume of sample used, the time course of the reaction, and the molar extinction coefficient of NAD⁺ (6.22 × 10³) at 340 nm.

The sera we used were from both outpatients and inpatients; however, their case histories were not available to us. The normal range for LAD was determined for sera that had no values outside the normal range in the usual SMA 12/60 (Technicon Instruments Corp., Tarrytown, N. Y. 10591) profile.

Results

The LAD assay described has an absolute requirement for DTT, NAD⁺, and enzyme (serum). The background reduction of NAD⁺ in the absence of lipoamide (lipoamide blank) is generally negligible. The highest LAD activity assayed to date had a net value of 473 kU/liter and the lipoamide blank for this serum was 0.3 kU/liter. Our results lead us to estimate the normal range for LAD in serum to be 3–19 kU/liter, based on results for sera from 100 patients.

¹ Nonstandard abbreviations used, and enzyme identification: LAD, lipoamide dehydrogenase (NADH) [NADH;lipoamide oxidoreductase, EC 1.6.4.3]; DTT, dithiothreitol; and Tris, tris(hydroxymethyl)aminomethane.

Received Sept. 1, 1975; accepted Nov. 17, 1975.
The optimum pH for LAD assay is 7.8 (Figure 1). Background activity is also least at this pH. LAD is inhibited by concentrations of Tris buffer higher than that recommended here (Figure 2). Hemolysis does not interfere.

The assay conditions described here are optimal for measuring LAD at concentrations up to 100 kU/liter (0.300 \( \Delta A_{340/10} \) min). Within these limits the reaction rate is directly proportional to the quantity of serum added (LAD activity) (Figure 3). Figures 4 and 5 demonstrate that the reaction is zero order with respect to the substrates, lipoamide, and NAD\(^+\), at the concentrations used. Also illustrated is the lessered (about an eighth) activity of the same sera with lipoic acid substituted for lipoamide. Under optimal conditions, within-day precision and day-to-day precision are satisfactory for both normal and abnormally high values (Table 1).

LAD activity is stable in fresh serum for 8 h at room temperature, for 24 h at 0-8 °C. Thawing and refreezing once does not significantly decrease the activity of LAD in serum. When a serum sample has been thawed it should be kept on ice until assayed.

Serum LAD activity was investigated in sera that had abnormally high values for either creatine phosphokinase [ATP:creatine phosphotransferase, EC 2.7.3.2]; aspartate aminotransferase [L-aspartate:2-oxoglutarate aminotransferase, EC 2.6.1.1]; alanine aminotransferase, [L-alanine:2-oxoglutarate aminotransferase, EC 2.6.1.2]; blood urea nitrogen; alkaline phosphatase [orthophosphoric monoester phosphohydrolase, EC 3.1.3.1]; lactate dehydrogenase [L-lactate:NAD oxidoreductase, EC 1.1.1.27]; or bilirubin (total, direct, and indirect). Figure 6 shows the statistically significant positive correlation between LAD activity and direct bilirubin concentration in 39 samples of serum (\( r = 0.435, P < 0.01 \)). Based on results for 15 or more samples each, we found that (possibly excepting alanine aminotransferase) the other serum analyses investigated do not correlate with increased serum LAD activity.

**Discussion**

Lipoamide dehydrogenase is an integral part of the multienzyme \( \alpha \)-ketol acid dehydrogenase complexes (2), where it functions to regenerate oxidized lipoic acid. The natural substrate for LAD is a reduced lipoic acid moiety that is covalently bound to the \( \epsilon \)-amino group of a lysine residue in one of the other component enzymes of the \( \alpha \)-ketol acid dehydrogenase complexes. In vitro, an analog, lipoamide, serves as a substitute for the protein-bound lipoyl moiety. The active (dihydro) form of the substrate is generated in slightly alkaline DTT solution (LAD II) before it is added to the assay mixture. By preincubating the serum sample with the NAD\(^+\) reagent (LAD I) the nonspecific background can be monitored before the reaction is started with LAD II. Routinely, background values are so low that this measurement is negligible. However, these data can be easily obtained without performing a separate assay. Previous reports have shown that LAD and diaphorase [NAD(P)H:
acceptor oxidoreductase, EC 1.6.99.-], a potential source of background interference, are identical proteins (3). It was subsequently shown, however, that diaphorase, an enzyme of no known physiological function, probably is a somewhat denatured form of LAD (4).

Most present enzymological methods for serum measure enzymes that originate and are localized either in the cytoplasm (e.g., alanine aminotransferase, lactate dehydrogenase) or in the mitochondria and the cytoplasm (e.g., aspartate aminotransferase). Methods for enzymes localized only in the mitochondrion—for example, glutamate dehydrogenase [L-glutamate:NAD+ oxidoreductase (deaminating), EC 1.4.1.2]—are fewer, and so studies on their diagnostic, prognostic, and monitoring potential have necessarily also been few (5). Enzymes localized exclusively within the mitochondrion should be of unique value for diagnosing acute disease, because tissue damage must be relatively severe before they would be expected to appear in the circulation.

Our method has given satisfactory results in daily use in a private medical-service laboratory without direct supervision from our laboratory. Although LAD activity is normally low or absent in serum, it is increased in certain pathological conditions. A positive correlation with direct bilirubin suggests a potential clinical value of serum LAD determinations, because increased values for direct bilirubin are always associated with liver or biliary tract disease (7).

Further studies are needed as to the normal range and clinical significance of serum LAD. We cannot at present pursue this investigation further, but we hope that this report will encourage other laboratories to do so.

We thank Mrs. Laurel Dingrando for expert technical assistance.

References