How Accurate Are Lactate Dehydrogenase Isoenzyme Estimations by the Thin-Layer Agarose Fluorescent Technique?

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We offer an assessment of the accuracy of the thin-layer agarose fluorescent technique of Elevitch et al. [Am. J. Clin. Pathol. 46, 692 (1966)]. We used semi-purified human lactate dehydrogenase isoenzymes 1 and 5. Both the lactate $\rightarrow$ pyruvate and pyruvate $\rightarrow$ lactate assays [Clin. Chem. 20, 1462 (1974)] appear to give, within the errors of the techniques used, a substantially unbiased estimate of both LD-1 and LD-5, although this must remain a provisional conclusion until a definitive method of assay for the total and isoenzymic LD activities is created. Introducing a buffer into the substrate mixture (lactate $\rightarrow$ pyruvate assay) had no effect on these findings except at extremes of pH, when marked inaccuracies occurred.

The accuracy of some analytical procedures in clinical chemistry can be established by the classical approach of calibration against highly purified standard materials, by analytical recovery experiments, and by the use of control materials of known composition. Although calibration materials for enzyme assays are not yet available, attempts should be made, even if only preliminary or provisional, to estimate the accuracy of the enzyme assays in common use today. Before discussing this topic further we should make clear that terms used in this article are defined in the Provisional Recommendations on Quality Control in Clinical Chemistry made by the Expert Panel (on Nomenclature and Principles of Quality Control in Clinical Chemistry) of the IFCC Committee on Standards (1). It is evident, from a study of these recommendations, that a true value may only exist when there is a reference method available for estimating an analyte. As there is no reference method for the assay of either lactate dehydrogenase [LD; EC 1.1.1.27] activity or of LD isoenzyme activities in human serum, it would seem impossible to determine the accuracy of any current LD isoenzyme procedure. However, a provisional estimate can be attempted if assigned values are used for LD isoenzyme activities.

That there is a need for such an assessment is best illustrated by comparing the results obtained by two separate methods. With electrophoresis on polyacrylamide (2), an insignificant amount of LD-5 activity is detected, whereas with electrophoresis on agarose appreciable amounts of this isoenzyme are detected (3). This is true whether the lactate $\rightarrow$ pyruvate or pyruvate $\rightarrow$ lactate detection technique is used (4). Which of these two methods, it can reasonably be asked, more accurately reflects the true value of the LD isoenzyme in the serum of a patient?

That any method has limitations is self-evident. Even when some or all of these limitations are recognized and allowed for by careful technique, it must still be stressed that this is no substitute for determining the accuracy of the method. Thus in determining LD isoenzymes by electrophoresis on thin layers of agarose the upper limits of the detection reaction must be carefully defined for each LD isoenzyme, because if it is exceeded in cases of abnormal or bizarre LD isoenzyme patterns the method fails (5). The use of quality-control procedures will establish the precision of the method (6) and extensive experience with the technique will alert one to sources of artefact such as renal disease (7) and hemodialysis (8), but the question of the true LD isoenzyme values still remains.

We have previously shown that similar LD isoenzyme values can be obtained for human sera whether pyruvate or lactate is used as substrate in the isoenzyme detection reaction (4), which suggests, but does not prove, that the methods have, at least, the same accuracy. We now wanted to estimate the accuracy more directly, by use of semipurified human LD isoenzymes (9). To do this, we estimated the individual activities of isolated LD-1 and LD-5 under optimum reaction rate assay conditions, mixed the two preparations together in a known proportion, and estimated the LD isoenzyme content, as a ratio of the two isoenzymes, after separation with the thin-layer agarose system.

Materials and Methods

Chemicals

All chemicals, except as noted, were obtained from the Fisher Scientific Co., Fair Lawn, N. J. 01410. 2-Amino-2-methyl-1-propanol, sodium pyruvate (Type II), lithium d,l-lactate (Grade DL-X; approximately 49.1% L-isomer), disodium $\beta$-NADH (Grade III), and $\beta$-NAD$^+$ (Grade III) were obtained from Sigma Chemical Co., St. Louis, Mo. 63178. $\beta$-NADH and $\beta$-NAD$^+$ were stored desiccated, in the dark at room temperature.
temperature and at \(-15^\circ C\), respectively. \(N,N\)-bis(2-hydroxyethyl)glycine, 1,4-piperazinediethanesulfonic acid, 3-[(2-hydroxy-1,1-bis(hydroxymethyl)ethyl)amino]-1-propanesulfonic acid and 2-amino-2-(hydroxymethyl)-1,3-propanediol were obtained from Calbiochem, La Jolla, Calif. 92037.

Sodium pyruvate solutions, either 20 or 10 mmol/liter, were prepared daily in phosphate buffer (0.1 mol/liter, pH 7.4 at 20 \(^\circ C\)) and immediately before each assay were used as the diluents in preparing a 423 \(\mu\)mol/liter solution of NADH.

\(D,L\)-Lactate solution, 1.0 mol/liter, was adjusted to about 7.0 (20 \(^\circ C\)) and immediately before each assay was used as the diluent in preparing an NAD\(^+\) solution of 7.5 mmol/liter.

Barbital buffer, ionic strength 0.05, was prepared from 10.3 g of sodium diethylbarbiturate, 0.35 g of disodium (ethylenedinitrilo)tetraacetate, adjusted to the required pH (see below) before use with 1 mol/liter HCl, and diluted to 1 liter with doubly distilled water.

Materials

\textit{Agarose Universal Electrophoresis Film} was from Corning Medical Diagnostics, Medfield, Mass. 02052, and Cellulose Polycarbonate Membranes from Gelman Instrument Co., Ann Arbor, Mich. 48106.

\textit{Validate Quality Control Sera} (lot No. 0321034) was supplied by Warner-Chilcott Laboratories, Scarborourth, Ont., and was reconstituted according to the manufacturer's instructions.

Tissue

Human liver and heart tissues were obtained from routine autopsies and stored at \(-80^\circ C\) until required. The tissue was allowed to thaw on ice and homogenized in ice-cold 20 mmol/liter 2-amino-2-(hydroxymethyl)-1,3-propanediol hydrochloride buffer (pH 7.4 at 20 \(^\circ C\)) and LD-5 and LD-1 were prepared from these homogenates by a two-step mini-column technique with diethylaminoethyl-Sephadex A-50 (9). LD-5 was finally suspended in the 20 mmol/liter 2-amino-2-(hydroxymethyl)-1,3-propanediol hydrochloride buffer; LD-1 was in the same buffer with the addition of 220 mmol of NaCl per liter. These suspensions were diluted to a suitable final enzymic activity with 150 mmol/liter NaCl. All preparations were checked for electrophoretic homogeneity before use in the accuracy experiments.

LD Isoenzyme Assays

The LD isoenzymes in 1 \(\mu\)l of material were separated by thin-layer electrophoresis on agarose (10) and detected by use of either (a) the isoenzyme reaction between L-lactate and NAD\(^+\) or (b) the reaction between pyruvate and NADH, as follows.

(a) \textit{With use of the L-lactate + NAD\(^+\) reaction.} Electrophoresis was performed at pH 8.6 (20 \(^\circ C\)), 10 V/cm, and 15–17 mA. After electrophoresis, an overlay of 20 drops of the substrate and isoenzyme solution was applied along the anode side of the agarose film and spread evenly across the film with the side of a pipette, in a single smooth motion. Excess solution was pushed off the gel by this procedure. The film was then incubated (moist chamber) at 38 \(^\circ C\) for 15 min in the system supplied by Corning Medical Diagnostics. The film was dried in the drying chamber (80 \(^\circ C\), which required 15–20 min, and NADH formation then detected by fluorescence (excitation 365 nm, emission 405 nm) with a Densicomp/Model 445-50 Fluorescence Recording Densitometer (supplied by Clifford Instruments Inc. but now available through Corning Medical Diagnostics). Peaks were quantitated by measuring peak heights (11).

This reaction was also done in the presence of the following buffers (0.5 mol/liter) over a range of pH values: 2-amino-2-methyl-1-propanol, \(N,N\)-bis(2-hydroxyethyl)glycine, 1,4-piperazinediethanesulfonic acid, 3-[(2-hydroxy-1,1-bis(hydroxymethyl)ethyl)amino]-1-propanesulfonic acid, and 2-amino-2-(hydroxymethyl)-1,3-propanediol (with and without addition of 5 mmol/liter disodium (ethylenedinitrilo)tetraacetate).

(b) \textit{With use of pyruvate + NADH reaction.} Electrophoresis on agarose was done at pH 9.1 (20 \(^\circ C\)) 10 V/cm, and 15–17 mA. Then a cellulose polyacetate membrane, soaked in the substrate and coenzyme solution and blotted dry, was laid upon the agarose film, care being taken to avoid entrapping air bubbles. The agarose and overlay films were incubated in the moist chamber (see above) for 5 min, the polyacetate strip was removed, and the incubation continued for 15 min. After drying, NADH utilization over the isoenzyme bands was detected densitometrically by using the "Linear Inverse" mode of operation.

LD Activity Assay

For assays we used a Model 8600 Reaction Rate Analyzer (LKB Instruments, Inc., Rockville, Md. 20852), and the pyruvate \(\rightarrow\) lactate assay at 37 \(^\circ C\) (12). The reaction buffer was 50 mmol/liter 2-amino-2-(hydroxymethyl)-1,3-propanediol hydrochloride (pH 7.4 at 37 \(^\circ C\)), containing 5 mmol of disodium (ethylenedinitrilo)tetraacetate and 150 \(\mu\)mol of NADH per liter of the mixture. The sample volume was 25 \(\mu\)l. The reaction was started with pyruvate at a concentration of either 1.2 or 3.5 mmol/liter of reaction mixture.

Results

\textbf{Optimum Conditions for Precision of the LD Assay for Quantifying LD-1 and LD-5}

We found the optimum concentration of pyruvate for the assay to be 1.2 and 3.5 mmol/liter for LD-1 and LD-5, respectively (Figure 1), using the test formulation of the Scandinavian Recommended Method for LD assays (12) except, of course, for the substrate concentration when assaying LD-5 (see \textit{LD Activity Assay}).

Each LD preparation was pre-diluted to have final activities in the range found for healthy subjects. This precaution was taken so that the capacity of the assay system would not be exceeded (5). Each preparation was assayed 10 to 20 times; the CV did not exceed 3% and
Fig. 1. The substrate optima for LD-1 and LD-5 at 37 °C
The assay system used is described in Materials and Methods. Semipurified human LD-1 (●-●) and LD-5 (○-○) had maximum activities of 620 and 454 U/liter, respectively. The arrows indicate the assay substrate concentration we subsequently used for assaying the respective isoenzymes.

Fig. 2. (a) The homogeneity of the isoenzyme preparations. (b) The separation of LD-1 and LD-5 in a mixture
The tracings show the fluorescent densitometric recording of the LD isoenzymes after detection of isoenzyme activity by use of the lactate + NAD+ reaction

5% for preparations of LD-1 and LD-5, respectively. The "true" value (i.e., the assigned value) for LD-1 and LD-5 activity in each preparation was assumed to be the mean value of these assay results.

Precision of the LD Isoenzyme Assays
Since September 1974 it has been the practice in this laboratory to put a suitable quality-control serum (6) in one position of the eight-sample agarose plate used for LD isoenzyme separations when using the conventional lactate → pyruvate assay. While the present work was in progress, we did 146 separate quality-control runs with the following standard deviations (as % of total LD activity) for LD-1 through LD-5: 1.08, 1.11, 0.62, 0.88, and 1.13, respectively. The quality-control serum had a "normal" LD isoenzyme pattern. Using the pyruvate → lactate assay (4), we obtained standard deviations of <2%, i.e., about twice those obtained with the lactate → pyruvate assay.

The precision of the scanning fluorescent densitometer recording system, measured by repeated scans of a single strip, had a CV of <0.5%, thus suggesting that the imprecision of the isoenzyme assay is not principally due to the densitometer.

Accuracy of the Estimation of LD Isoenzymes
Before each experiment the homogeneity of each preparation was checked (Figure 2a) and each mixture used showed LD activity only in the LD-1 and LD-5 regions with no evidence of hybridization (Figure 2b). The results of several separation experiments with different preparations are given in Table 1. From these data it can be concluded, that, within the error of both types of assay the assigned value is not significantly different from the observed values for either the lactate → pyruvate or pyruvate → lactate assays.

Effect of Buffering the Substrate on the Accuracy of LD Isoenzyme Estimations
Although the substrate is buffered in the pyruvate → lactate isoenzyme assay (4), it is not, in the lactate → pyruvate assay. It should be remembered that the electrophoretic medium contains a low concentration of barbital buffer (pH 9.1, 20 °C). In the latter assay, the 0.5 mol/liter lactate solution (with respect to the L-isomer) is adjusted to about pH 7 (20 °C). As the pKₐ (25 °C) of lactic acid is 3.86 (13), this reaction mixture possesses no buffering capacity at all. It is of interest that Corning Medical Diagnostics use a reaction mixture containing 0.5 mol of 2-amino-2-methyl-1-propanol per liter for their proprietary lactate → pyruvate detection reaction. We therefore decided to use several buffers over the pH (20 °C) range of 8.0 to 10.0 with the lactate → pyruvate system, to determine what the effect on

Table 1. Values of LD-1 Observed after Separating a Mixture of LD-1 and LD-5 of Known Proportions

<table>
<thead>
<tr>
<th>LD-1 in mixture, %</th>
<th>% LD-1 found after separation (mean ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lactate → pyruvate assay</td>
<td></td>
</tr>
<tr>
<td>44.7</td>
<td>46.6 ± 1.06 (n = 8)</td>
</tr>
<tr>
<td>53.2</td>
<td>52.3 ± 1.60 (n = 8)</td>
</tr>
<tr>
<td>63.0</td>
<td>62.5 ± 1.77 (n = 8)</td>
</tr>
<tr>
<td>Pyruvate → lactate assay with 20 mmol/liter pyruvate</td>
<td></td>
</tr>
<tr>
<td>51.2</td>
<td>48.5 ± 0.76 (n = 8)</td>
</tr>
<tr>
<td>with 10 mmol/liter pyruvate</td>
<td></td>
</tr>
<tr>
<td>51.2</td>
<td>52.0 ± 0.93 (n = 8)</td>
</tr>
</tbody>
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buffers in the assay medium does not alter the accuracy, except at extremes of pH, and there is one marked disadvantage to their use. In humid weather we found that the developed agarose plate rapidly became sticky as it took up moisture from the atmosphere. The plate then sticks to the matt black mask used for positioning and shielding the agarose plate. This effect is not encountered with our basic lactate substrate preparation. We therefore conclude that the simple reaction mixture of L-lactate and NAD+, without buffer, is adequate for producing accurate estimations of, at least, LD-1 and LD-5.

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References