Two Methods Compared for Measuring LD-1/Total LD Activity in Serum

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We present evidence for the utility of an improved assay for the activity of lactate dehydrogenase (EC 1.1.1.27) isoenzymes 1 and 2 in serum, involving inhibition of the H-subunit of LD by pyruvate at pH 7.1. Results correlate well with the LD-1/total LD ratio as evaluated by immunological assay and, as an index to infarct, the method is superior to either the change in CK-MB activity or to the LD-1 activity or to a combination of these tests, as is the percentage of LD-1 to total LD activity. Moreover, the percentage inhibition of LD activity by pyruvate may have an advantage over other methods of isoenzyme fractionation because of its smaller population CV for patients with acute myocardial infarction than is true of other methods. We also demonstrate how, using a linear discriminant analysis, we compared this method with alternative methods. We determined that evaluation of CK-MB isoenzyme contributes no information in addition to that obtained from the LD-1 isoenzyme.

Additional Keyphrases: LD-1/total LD ratio vs percent inhibition of lactate dehydrogenase by pyruvate • myocardial infarct • creatine kinase isoenzyme MB

Studies from this laboratory (1–3) have indicated that lactate dehydrogenase (EC 1.1.1.27) isoenzyme-1 (LD-1) activity in serum can be determined by using an assay that exploits the superiority of the pyruvate-to-lactate reaction (4) that is optimized at two concentrations of pyruvate.1 Moreover, the importance of determining LD-1 activity has been reinforced by the observation that it increases during the first 12 h after myocardial infarction and even before creatine kinase (CK; EC 2.7.3.2) isoenzyme MB activity reaches its maximum (5–8), although LD-1 currently is not relied upon as much as CK-MB is for confirming the diagnosis of acute myocardial infarction.

That the importance of measuring LD-1 isoenzyme has been underestimated and that its role is not clear (7) is illustrated by studies such as the one supporting measurement of LD-1 activity by the α-hydroxybutyrate dehydrogenase (HBD) method, along with CK-MB, on two consecutive days (9), thus minimizing the importance of the assay or its timing because the combination of tests was considered adequate for clinical use. One may then ask whether the adequacy of CK-MB assay perceived by clinicians accounts for a different attitude toward LD-1 isoenzyme being held by clinicians and by chemicalists. Jablonsky et al. (7) called attention to a lack of clinical interest in LD-1 isoenzyme because of misperceptions among cardiologists about the methodological and clinical status of this test, but it is not clear from reviews (10, 11) whether the dissatisfaction is related to problems of the logistics of performing the evaluation and reporting results; difficulty in interpreting a high cutoff value, resulting in a specificity that is not clinically useful (isoenzyme-1/isoenzyme-2 ratio); or to analytical measurements that are clinically unacceptable or not acceptable as compared with CK-MB. The accuracy of measuring the percentage of LD-1/total LD or the LD-1/LD-2 ratio for the early detection and diagnosis of acute myocardial infarction (AMI) is well documented (6, 7, 12). However, a misperception about its accuracy does not itself appear to be the major reason for disinterest in LD-1.

Despite numerous studies defining the efficacy of CK-MB and LD-1 methods, there still is no consensus concerning the advantage of any approach over another, except for the fact that electrophoresis is widely used for CK-MB or LD-1 analyses (13). In addition, when the isoenzyme-1/isoenzyme-2 ratio is measured by electrophoresis it is interpreted by use of an inappropriate standard (12). Results by column chromatography (14) and electrophoresis have been comparable, but there have been continuing reports of assays by direct spectrophotometric kinetic assays (15, 16). Procedures for separating LD-1 have been based on differences in physical or kinetic properties of the H- and M-type LD, such as heat denaturation, stability of urea (17), inhibition by oxamate or oxalate (18), differences in Michaelis constant (19–22), and differences in the immunological properties of the LD isoenzymes (20, 23). The principal advantage of kinetic methods for determining LD isoenzyme content is their rapidity, their inexpensiveness, and their adaptability to automated instruments.

Here we report on further studies in which we measured the H-subunit content of LD activity in serum (2), comparing this method with the immunological assay for LD-1 (23). The method involves two LD assays under different conditions, and does not require separation of the LD-1 fraction before assay. We also construct functions, using the results of CK-MB and LD-1 concentrations measured in serum, to determine their efficiency in classifying patients with and without infarcts, and we rank these tests according to the contribution of each as single or joint predictors in classifying patients.

Materials and Methods

Enzyme Assays

Isoenzymes were quantified with kits for immunological inhibition of CK-MB (SmithKline Beckman Corp., Brea, CA) and for immunological assay of LD-1 (Isomune; Roche Diagnostics, Nutley, NJ). These were compared with the assay of LD-1 activity by the method of optimized pyruvate inhibition (2) with kits manufactured for Bridgeport Hospital by Diagnostic Chemicals, Ltd, Monroe, CT. Human H4, H3M, and M4 isoenzymes of LD were purchased from Sigma Chemical Co., St. Louis, MO.

We carried out serial CK-MB analyses at 6-h intervals during the first 24 h after admission of the patient and LD-1 analyses at the peak of CK-MB activity or 6 h thereafter as previously described (8). For immunochemical LD-1 assay, performed at 30 °C, we used a Cobas-Bio centrifugal analyz-
er (Roche Instruments, Nutley, NJ).

LD-1 inhibition was at pH 7.1 and 30 or 37 °C by the method of Bernstein and Everette (2), under the following conditions. Total LD activity is measured in a 0.34 mmol/L solution of the oxidized substrate. This is followed by a second assay in a reagent containing, per liter, 0.2 mmol of NADH and 5 mmol (30 °C) or 5.5 mmol (37 °C) of pyruvate, respectively. The NADH concentration was increased (from 0.14 mmol/L) for the clinical studies to eliminate substrate depletion at high LD activities, and the standard curve for the reaction is linear to >500 U/L. The modification of pyruvate concentration at 37 °C is an empirically determined adjustment for the increased activity at the higher temperature.

Subjects

The 86 patients in this study were admitted to Bridgeport Hospital for diagnosis or exclusion of AMI. The diagnoses were established by clinical presentation, electrocardiography, serial enzyme measurements, and additional catheterization and angiographic studies as required.

After grouping the patients into two categories on the basis of a detailed study of the medical records, we carried out data analyses, using the "Statistical Package for the Social Sciences." The mean age of the study population was well over 60 years, but congestive heart failure was not a common complication, although it is the most significant factor determining which patients will require an extended stay in the hospital. In the absence of arrhythmias these patients' cases are considered uncomplicated unless pulmonary infiltrates appear in their chest roentgenograms, appropriate clinical findings are present, and (or) they have decreased ejection fractions, any of which would lead to their condition being classified as in Killip Class III or IV. LD-5 is massively increased in patients who develop cardiogenic shock as a complication of AMI, but there were no patients in Killip Class IV in this study, which would have been complicated by any effect of severe congestive heart failure on the LD-1 isoenzyme in their serum. We carried out basic statistics to measure central tendency, variation, and skewness, and plotted histograms by group for each variable. This was followed by one-way analyses of variance and discriminant analyses, to determine the best model for predicting membership in either the AMI or non-AMI groups.

Results

Figure 1a, a plot of \( K_m \) for pyruvate vs pH for both the \( H_4 \) and \( M_4 \) isoenzyme of LD, shows the decreasing affinity of LD for pyruvate with increasing pH at 7. Figure 1b is a plot of the percentage of maximum activities of \( H_4 \) and \( M_4 \) in human LD as a function of pyruvate concentration at pH 7.0. On the basis of these data, we chose a pair of pyruvate concentrations for assay for LD isoenzyme composition, utilizing the pH-dependent inhibition of LD activity by pyruvate to distinguish the activities of the isoenzymes at defined pH and temperature. Measuring LD activity after the inhibition of LD activity of human \( H_4 \), \( H_2 M_4 \), and \( M_4 \) in different combined ratios gives a parabolic relationship between the percent H-subunit activity and percent inhibition \( (r = 0.8957) \).

We carried out further studies of the 32 AMI and 53 non-AMI patients to compare the percentage of LD-1/total LD activity and percent inhibition of LD activity (percent INH). The percent INH and percent LD-1 are correlated \( (r = 0.876) \) by the equation:

\[
\%\text{INH} = 25.0 + 0.61378 \times (\%\text{LD-1})
\]

The comparison of AMI and non-AMI patients by assay at 30 °C for inhibition of LD activity in the presence of 5.0 mmol of pyruvate per liter showed the population coefficient of variation for percent inhibition to be considerably lower than for percent LD-1 isoenzyme, as follows: for AMI, % LD-1 = 26.26%; %INH = 6.93; for non-AMI, % LD-1 = 33.85%; %INH = 16.37%. Similarly, when the assay was modified for assay at 37 °C (5.5 mmol of pyruvate per liter) we obtained an overall population CV of 52.22% for percent LD-1 as compared with 25.98% for percent INH.

In Table 1 we compare the patients' results obtained by inhibiting LD activity at 37 °C by use of 5.5 mmol/L pyruvate with the measurements of CK-MB activity and percentage of LD-1/total LD activity in the AMI and the non-AMI groups. The standard deviation for the populations was higher for CK-MB and LD-1 activity than for either percent LD-1 or for percent INH. This proved to be important in our other studies.
Table 1. Percent LD-1 as Measured by Two Methods

<table>
<thead>
<tr>
<th>Diagnosis</th>
<th>CK-MB, U/L</th>
<th>%LD-1</th>
<th>Inhibiton</th>
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<tr>
<td>Trauma</td>
<td>20</td>
<td>24</td>
<td>13</td>
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<tr>
<td>Gunshot</td>
<td>52</td>
<td>14</td>
<td>30</td>
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<tr>
<td>Trauma</td>
<td>36</td>
<td>15</td>
<td>18</td>
</tr>
<tr>
<td>Angina</td>
<td>62</td>
<td>3</td>
<td>22</td>
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<tr>
<td>CHFV, FIB</td>
<td>46</td>
<td></td>
<td>43</td>
</tr>
<tr>
<td>Cirrhosis, thrombocytopenia</td>
<td>71</td>
<td></td>
<td>8</td>
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<tr>
<td>Angina</td>
<td>65</td>
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<tr>
<td>CHFc</td>
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*LD-1 increase to 33% represents early evolving AMI. *Anterolateral AMI with absent CK-MB and LD-1 changes. Total LD activity (0.34 mmol/L; P<0.1) increased. *Congestive heart failure.

In addition to basic statistics, we carried out one-way analyses of variance and established that the differences between the two groups, AMI and non-AMI, were statistically significant (p<0.01) for all of the independent variables: LD-1, percent LD-1, and percent INH. The F-value for percent LD-1 was the highest at 160.6. The correlation matrix for these predictors showed the highest correlation between: LD-1 activity and LD activity (pyruvate); LD-1 and percent LD-1; and percent LD-1 and percent INH. We also conducted discriminant analyses, with results significant at the 0.01 level.

In Table 2 we compare results of discriminant analyses that best classify patients, using the candidate predictors. We entered the tests one at a time to determine combinations of the fewest predictors that would result in low misclassification rates and to determine the contribution of each predictor to the classification functions. The very high eigenvalue, high canonical correlation, high chi square, and low Wilk's lambda for the percent LD-1 shows good separation with this single predictor variable, somewhat better than the results obtained with a combination of total LD activity and its inhibition by pyruvate. However, we obtained the best separations in any combination that included LD-1/total LD activity, even using inhibition of subunit content of LD activity for proportion of LD-1 activity. Additionally, the change in CK-MB was found to contribute no additional predictive value in distinguishing the AMI and non-AMI groups, mainly because of shared variance with the other predictors.

The results clearly show the discriminating power of percent LD-1 and reinforce the principle of measuring the total LD activity whenever isoenzyme-1 LD activity is obtained. Although the results show that the discriminating power of percent INH with LD activity measured with pyruvate as substrate is not as great as that of percent LD-1, the separation is more than adequate.

Discussion

We conclude that measuring LD-1 either as a percentage of isoenzyme-1/total LD (6) or as percent inhibition of H-subunit activity is superior to measuring the activity of LD-1 or CK-MB, either individually or in combination, an important conclusion in view of the current reliance on serial CK and CK-MB isoenzyme measurements in many institutions. Indeed, the findings make their analysis extremely difficult to explain or justify the declining use of LD isoenzyme-1 evaluation for the diagnosis of AMI (7). Perhaps there are factors related to untimely reporting rather than accuracy of laboratory results that have provoked this disinterest in LD-1. Moreover, there is a need for early recognition of AMI in order to initiate treatments to limit the progression of infarct and avoid late complications. This has created greater interest in excluding non-AMI patients who have equivalent CK-MB changes.

There is a lack of agreement about the best method for measuring LD-1, but the studies of Leung and Henderson (12) provide some explanation for the popularity of electrophoresis over all other methods. The value of electrophoretically separating CK-MB and LD-1 may be compared with that of measuring CK-MB immunologically combined with HBD on the first and second day (9), but the better efficiency of such an approach has not adequately been validated with respect to errors of sensitivity estimations (26). Nevertheless, the study (9) supports measuring the activity of LD-1 after 12 h and kinetically, mainly because it is used with CK-MB isoenzyme in addition to electrocardiographic and clinical criteria. Moreover, the study (9) places greater importance in the value of LD-1 used for a late marker for AMI than in the method of the LD-1 assay.

We present evidence for the adequacy of an LD-1 assay that is nearly as accurate as the immunological LD-1 assay but is as inexpensive as the HBD assay. The advantages of this approach have not been previously identified. Other assays have recently been reported for measuring the activities of LD-1 and LD-2 isoenzyme by automated kinetic procedures (15, 16). While we do not report as high a correlation with inhibition by pyruvate and LD-1/total LD determined by immunological assay as that reported for alkaline inactivation, the manipulation required in the latter method is as great as that required for immunological assay. We here substantiate by its clinical performance the use of substrate inhibition for measuring LD-1 activity. This kinetic method for determining the isoenzyme contents of LD activity is based on the fact that H-type LD is significantly inhibited by high concentrations of pyruvate (1).
associated with the formation at pH 7 of an abortive complex between enzyme, NAD\(^+\), and pyruvate. This assay takes advantage of the known superiority of the pyruvate-to-lactate reaction (3), and it is simpler than those recently reported (15, 16).

The comparison of the pyruvate-inhibition assay with the immunological assay for LD-1 fraction shows a slight superiority of the latter despite the lower CVs of the inhibition method within the study populations. The differences between these methods are largely found in examining the means and variances between the AMI and non-AMI populations. These differences may not be substantiated in studies with a larger population. The measurement of LD-1 by inhibition of LD subunit activity is dependent on a set of variables that includes the differences in \(K_m\) for pyruvate of the H and M subunits. The means of LD isoenzyme inhibition in non-AMI and patients with small AMI tend to cluster in a range of between 45 and 48% inhibition. Comparable results in the LD-1 immunological assay would be consistently less than 29% of the total LD activity. This would be similar to the population with an isoenzyme-1/isoenzyme-2 ratio in a range from 0.60–0.85 by electrophoresis, currently under investigation by Henderson and coworkers (7, 12). For this group it becomes very difficult to assess the accuracy of these methods because of the limited size of the subpopulation under investigation. However, reduction of the population variances becomes a desirable feature in determining their sensitivities and for comparing the performance of these methods in the clinical subpopulation of borderline AMI patients. The method of inhibition of subunit content, though it is biased in estimating the H-type LD activity because it is affected by the presence of LD-2 isoenzyme, does appear to accurately measure differential increases in LD-1 isoenzyme activity in serum.

What significance is there to the distinct and subtle differences between the immunological and the enzymatic assay for LD-1 isoenzyme composition? The results suggest that the significance is more apparent than real, as judged from the extremely good efficiency obtained with either method. The apparent problem of concern with either method has been the issue of dealing with LD-1 activities in the presence of congestive heart failure. This only applies to the patient with Killip Class II–IV with reduced ejection fractions and severe circulatory impairment associated with increased LD-5 activity in cardiogenic shock or with increased LD-2 and-3 isoenzyme in some patients with pulmonary edema. When patients are found to have a total LD activity that is out of proportion to the LD-1 activity by any measurement, they are usually found to have an increased LD-5 activity associated with generalized hypoperfusion.

In these patients there is no need for a superior LD-1 assay because of the likelihood that there are electrocardiographic and clinical findings consistent with AMI with massive increase in CK-MB isoenzyme. The HBD would also be high because of massive increases in LD activity in the serum, originating not only from the heart but also from the liver. The most important clinical problem for the patient is management of cardiogenic shock.

The present studies confirm the validity and discriminating power of LD-1 subunit content measured as percent inhibition of LD activity by pyruvate. More importantly, they demonstrate how we can evaluate these tests and establish the contribution of each to a vector of observations in forming a joint predictor for classifying patients. We found that, by comparison with LD-1 activity as percent of total LD activity or as percent inhibition, CK-MB and LD-1 activities provide no additional discrimination beyond that of percent inhibition because of the large population variance of enzyme activities in the AMI and non-AMI populations. Finally, we think that the more rapid and accurate analyses of LD-1 here demonstrated may contribute to the improved treatment of AMI because of the current emphasis on more rapid diagnosis and treatment to reduce late complications, especially in those patients who have small infarcts with equivocal changes in the concentration of CK-MB activity in their serum.

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References


