Micromethod for Determination of Lactate Dehydrogenase Isoenzyme C4 Activity in Human Seminal Plasma

Gerald P. Butrimovitz, Francis Farina, and Ira Sharlip

We have developed an automated enzymatic assay for LDH-C4, a lactate dehydrogenase (EC 1.1.1.27) isoenzyme found exclusively in spermatozoa, as a marker of human spermatogenesis. LDH-C4 activity is assayed bichromatically in the Abbott ABA-100 spectrophotometer, with 2-oxohexanoate as substrate. As many as 27 samples (2.5 µL) of human seminal plasma can be analyzed sequentially, with apparent zero-order kinetic conditions and with a precision (CV) of 5%. The apparent Km for the 2-oxohexanoate reaction is 11.2 mmol/L. Electrophoretic and enzymatic studies indicate that LDH-C4 acts on this substrate to produce hydroxyhexanoate, in contrast to lactate dehydrogenase isoenzymes 1 and 5—further evidence for the uniqueness of LDH-C4. The sensitivity, specificity, and speed of this assay system makes it practicable for studies on, and evaluation of, male fertility.

Additional Keyphrases: spermatogenesis · infertility · effectiveness of vasectomy reversal

In most human tissue, lactate dehydrogenase (LDH, EC 1.1.1.27) is present as a series of five tetrameric isoenzymes, each assembled from two types of polypeptide unit: H (heart) and M (muscle) (1). In 1963, Blanco and Zinkham (2) electrophoretically identified a sixth LDH isoenzyme, LDH-C4, confined to the testes and sperm. Since then, kinetic (3), molecular (4), and immunological studies (5) have provided further evidence for the unique nature of LDH-C4 and its association with maturation, motility, and survival of spermatozoa. Changes in activity of LDH-C4 in the testes parallel the development or loss of seminal elements (6, 7). Measurement of total LDH in semen has no diagnostic value (8), but the unique catalytic properties of LDH-C4, probably ascribable to its testicular origin, afford an opportunity to develop a selective enzymatic assay for it (3). Allen (9) and Burgos et al. (10) have demonstrated that only LDH-C4 acts on the substrate 2-oxohexanoate, but no convenient procedure was yet available to the clinical laboratory for measuring LDH-C4 in seminal plasma. We have developed such a method.

Materials and Methods

β-NADH (grade I) was obtained from Boehringer Mannheim Biochemicals, Indianapolis, IN 46250. 2-Oxohexanoate (α-keto-caproic acid, sodium salt), LDH-1 (H4 human, type XIX, 98% homogeneous), and LDH-5 (M4 human, type XXVIII) were from Sigma Chemical Co., St. Louis, MO 63178. Dibasic sodium phosphate was from J. T. Baker Co., Phillipsburg, NJ 08865, and monobasic potassium phosphate from Mallinkrodt, Inc., St. Louis, MO 63134. Sodium hydroxide was used to adjust the pH of the various solutions.

Supernates of seminal plasma were prepared from twice-frozen (at -20°C) semen samples that were allowed to thaw at room temperature, then centrifuged at approximately 11 000 × g for 2 min (11).

For enzyme assays we used an ABA-100 discrete analyzer (Abbott Labs., Dallas, TX 75247) operated at 30°C, with a 340/380 nm bichromatic filter. Samples of seminal plasma, 2.5 µL, are diluted with a 0.51 mmol/L solution of NADH in phosphate buffer (pH 7.4, 0.1 mol/L), by use of a 1:201 diluter ABA-100 syringe plate. After a 40-min incubation, 50 µL of 2-oxohexanoate (50 mmol/L of phosphate buffer) is added to the reaction cuvet with an SMI Micropettor (cat. no. 1058D; Scientific Manufacturing Industries, Emeryville, CA 94608). A pre-incubation delay of 40 s permits a "first revolution" reading in the normal kinetic mode (starting at cuvet no. 5). First- and second-interval 5-min absorbance or activity reading averages are reported.

For electrophoretic assays we used a Bio-Rad cell system (no. 1415; Bio-Rad Labs., Richmond, CA 94804) at 13°C and 400 V. With it, 1-µL samples of seminal plasma supernates were electrophoresed on Universal Agarose Film for 35 min and stained with the LD-substrate system (both from Corning Medical, Medfield, MA 02052).

Results

Optimization of assay. We found that under conditions established earlier (10) for crude lysates of spermatozoa there was a significant deviation from zero-order conditions during the course of reaction. To overcome the rapid loss of enzyme activity during the 5-min measurement period, we used excess NADH (0.46 mmol/L, final concentration) and included a 40-min pre-reaction incubation, which removes endogenous substrate such as pyruvate (12). These steps eliminate a potential positive error of between 109 and 414 U/L (Table 1) for representative semen samples with normal and low sperm count.

Using these optimized conditions, we studied the effect of 2-oxohexanoate concentration on LDH-C4 activity (Figure 1). Activity was maximum at a 2-oxohexanoate concentration of 37.5 mmol/L. However, to maintain the linear relation with absorbance for samples with high activity, we used a concentration of 60 mmol/L in the assay. The shape of the curve in Figure 1 indicates 50% inhibition of LDH-C4 activity when the 2-oxohexanoate is increased to 100 mmol/L. Lineweaver–Burk reciprocal plots (data not shown) were linear for the ascending portion of the hyperbola for various human semen specimens, from which we calculated an apparent Km of 11.2 mmol/L for this reaction.

Linearity of reaction. Under the stated optimized conditions, the reaction showed apparent zero-order kinetics. Monitoring the assay reaction at 5-min intervals for as long as 30 min showed that the activity remained constant. Results were similar for samples of seminal plasma with LDH-C4 activities as great as 1500 U/L. For samples with activity to 3000 U/L, linearity is maintained (i.e., the substrate is not depleted) for the first 15 min of reaction. Evidently the forward reaction is not inhibited by formation.
Table 1. LDH-C₄ Activity in Human Seminal Plasma before and after Incubation (40 min, 30 °C)

<table>
<thead>
<tr>
<th>Before incubation</th>
<th>After incubation</th>
<th>Difference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Activity, U/L</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1132</td>
<td>1471</td>
<td>239</td>
</tr>
<tr>
<td>290</td>
<td>497</td>
<td>207</td>
</tr>
<tr>
<td>89</td>
<td>182</td>
<td>96</td>
</tr>
<tr>
<td>1472</td>
<td>1618</td>
<td>146</td>
</tr>
<tr>
<td>258</td>
<td>522</td>
<td>264</td>
</tr>
<tr>
<td>915</td>
<td>1329</td>
<td>414</td>
</tr>
<tr>
<td>101</td>
<td>292</td>
<td>191</td>
</tr>
<tr>
<td>70</td>
<td>233</td>
<td>163</td>
</tr>
<tr>
<td>238</td>
<td>436</td>
<td>198</td>
</tr>
<tr>
<td>1549</td>
<td>1820</td>
<td>271</td>
</tr>
<tr>
<td>38</td>
<td>190</td>
<td>152</td>
</tr>
</tbody>
</table>

Fig. 1. Lactate dehydrogenase-C₄ activity of human seminal plasma supernates
Averages of first and second 5-min absorbance readings of five separate samples, expressed as percentages of maximal activity, determined at eight concentrations of 2-oxohexanoate of the product, 2-hydroxyhexanoate (3). Data were similar for the same time interval at lower 2-oxohexanoate concentrations, but the activity was not maximal.

Linearity with dilution and precision. To assess variation in the assay due to the combined effects of dilution, mixing, addition, and spectrophotometric stability, we sequentially assayed two pools of human seminal plasma 10 times. For the high-activity pool the mean activity was 1666 U/L (CV 3.1%), for the low-activity pool the mean was 381 U/L (CV 5.1%) (Table 2). Figure 2 shows the precision of the assay over a broader range of activity: the dilution study indicates highly significant linear regression of \( r^2 = 0.999 \) \((p < 0.001)\), to 0.065 A/min, which represents an activity of 2800 U/L as measured by this method. Changes in the concentration of the enzyme thus appear to be directly proportional to changes in its measured activity. Preliminary studies also indicate a positive correlation \((r = 0.92)\) between enzyme activity and sperm count \((0 \text{ to } 130 \times 10^6/\text{mL}, n = 13)\) in human seminal plasma.

Specificity. The specificity of the assay was investigated enzymatically and electrophoretically with lactate, a substrate for all LDH isoenzymes (10), as the substrate. As shown in Figure 3, LDH-C₄ is absent in azospermia (Figure 3a) but is a major component of the total LDH activity in normospermia (Figure 3b). Commercial LDH-1 and LDH-5 preparations (Figure 3, c and d) did not have any catalytic action on oxohexanoate in this enzyme assay (Table 2) but did act upon a lactate substrate, as did LDH-C₄ (Table 3). Moreover, preparations of human seminal plasma diluted with buffer, LDH-1, or LDH-5 retained relative activities of 99 to 104% in relation to the seminal plasma pool (Table 2), indicating that there is no interaction between non-C₄ isoenzymes and the 2-oxohexanoate substrate.

Seminal plasma samples from five vasectomized men contained LDH-C₄ activities ranging from 0 to 138 U/L (mean, 42 U/L). This corresponds to about 2% of the activity found in normal human seminal plasma and supports previous reports that spermatozoa are present in the seminal duct above the site of vasectomy for as long as six months after vasectomy (13, 14). Electrophoretic studies suggest that the LDH in the ducts is in fact LDH-C₄, although such an interpretation must be made cautiously because of the much lower sensitivity of the electrophoretic methods.

Discussion

Other than microscopic examination of semen, there are few techniques for evaluating sperm quality (15). In light of reports of a nationwide decline in sperm count in men (16), advances in surgical vasovasotomy microtechniques (17),

![Graph showing % Maximum Activity vs. Oxo-hexanoate, mmol/L](image)

![Graph showing ΔA/min. x 10⁻³ vs. Dilution](image)
and an interest in the effectiveness of new contraceptives for males (18, 19), rapid quantitative methods for assessment of fertility are needed. Human seminal plasma represents one of the most complex fluids in the human body. Many of its proteins are unique to semen, and Bishop (20) has suggested that its biochemical assay may provide a "fingerprint"—that is, a unique marker of the individual. Because many seminal enzymes are 50-100-fold more active in human semen than in blood plasma, microliter samples can be analyzed with high sensitivity (21). Assay of LDH-C4 accurately reflects differentiated spermatogenic cells, especially late primary spermatocytes and spermatids (22).

Manual procedures for assay of enzymes in semen are cumbersome, time consuming, and not well suited to the clinical laboratory. Thus the instrumentation we selected for use in this study had to satisfy three criteria: (a) a positive-displacement sample-dilution system, suitable for highly viscous seminal fluid, (b) a flexible but automated kinetic data processor, and (c) a spectrophotometer giving stable measurements over a wide absorbance range. The ABA-100 can sample 2.5 μL of seminal plasma and dilute it 200-fold with a CV of <5.1%. This is particularly important in the evaluation of infertile patients, whose seminal samples may be limited to a few hundred microliters.

Burgos et al. (10) described an analysis for LDH-C4 in sperm lysates in which initial rate velocity was measured. We find this approach unacceptable because exogenous pyruvate-like substances are present in seminal plasma, which can yield falsely increased activities (Table 1). Such enzyme measurements, though perhaps well suited for purified extracts of sperm containing up to 80% LDH-C4 (3), are not well suited for sperm lysate in the presence of these competing NADH-reducing systems in seminal plasma. As is frequently suggested (12), the sample must be incubated if the kinetics are to be stable or zero-order. Use of a higher NADH concentration (0.41 mmol/L) than suggested by Burgos et al. (11) not only promotes reduction of pyruvate in the preliminary incubation step but also extends the assay to a wide range of enzyme activities. Reading absorbance at multiple time intervals permits evaluation of kinetic parameters and assures nondepletion of substrate. Double-reciprocal plots for analyses on normal human samples yielded a \( K_m \) of 11.2 mmol/L, at 30 \(^\circ\)C in excess NADH, as compared with 0.8 mmol/L at 37 \(^\circ\)C reported earlier (10). Possibly the higher catalytic activity achieved by use of preincubation was ascribable in part to endogenous pyruvate, for which the \( K_m \) for LDH-C4 is 114 μmol/L (10).

The precision of this system is about that for LDH determination in blood plasma (23). Because initial absorbance readings for diluted seminal plasma exceed 2.0, a stable and linear spectrophotometer must be used. Within this absorbance range, CVs are ≤5.1%, and dilution studies, which indicate a highly significant relation between enzyme concentration and enzyme activity, indicate that the assay is applicable to a wide range of clinical studies.

Bianco and Zinkham (2) suggested that LDH-C4 is composed of four subunits that differ from the A(M) and B(H) monomers. More recently, Goldberg (5) showed immunological individuality of LDH-C4 in sperm. Further studies (3, 10) continue to suggest that LDH-C4 is a distinct molecular form with a unique substrate affinity and kinetics. The present study on various forms of LDH supports the hypothesis for the isospecificity of 2-oxohexanate for LDH-C4. It is reasonable to assume that a lack of reactivity of LDH-1 and LDH-5 with 2-oxohexanate precludes reactivity with H,M hybrid tetramers. A striking finding was the limited reactivity with the substrate of LDH-C4 in the seminal plasma of vasectomized men. The high sensitivity of the system might thus be useful to reveal successful reversal of vasectomy, perhaps even before spermatozoa are detected microscopically.

This work was supported by BRSG Grant SO7 RR05355 awarded by the Biomedical Research Support Program, Division of Research Resources, NIH, by Abbott Laboratories, and by Gerber Products Company. We thank Dr. Ananda S. Prasad, Philip Lee, and Sheldon Margen for guidance throughout this work.

### Table 3. Substrate Affinities for LDH Isoenzymes

| Activity, U/L | Oxohexanate substrate | Lactate substrate *
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>LDH-1 preparation*</td>
<td>—</td>
<td>220</td>
</tr>
<tr>
<td>LDH-5 preparation*</td>
<td>—</td>
<td>190</td>
</tr>
<tr>
<td>LDH-C4 from seminal plasma</td>
<td>766</td>
<td>1287</td>
</tr>
</tbody>
</table>

*See Table 2 for details.

*Source: ref. 11.

### References


23. Package insert: LDH Clinical Chemistry Reagent, Abbott Laboratories, Diagnostic Division, South Pasadena, CA 94103.