Creatine Kinase and Lactate Dehydrogenase: Stability of Isoenzymes and Their Activity in Stored Human Plasma and Prostatic Tissue Extracts and Effect of Sample Dilution

Sydney A. Shain, Robert W. Boesel, Robert W. Klipper, and Chris M. Lancaster

We examined the effect of storing human plasma or extracts of prostate at −90 °C on the activity of creatine kinase and lactate dehydrogenase and isoenzyme distribution. Enzyme activities were unaltered during storage for as long as six weeks. If these preparations were thawed only once at 2 to 4 °C, they could be stored for as long as 165 days at −90 °C with no change in isoenzyme distribution. Inexplicably, apparent isoenzyme distribution of prostatic lactate dehydrogenase was sensitive to sample dilution, whereas the isoenzyme distribution of lactate dehydrogenase in plasma was not. Our observations emphasize the importance of validating details of analytical protocols that are to be used for quantification of new types of specimens.

Additional Keyphrases: variation, source of enzyme activity

Stability of creatine kinase (CK; EC 2.7.3.2) or lactate dehydrogenase (LDH; EC 1.1.1.27) activity depends on both storage temperature and the composition of the medium (1–7). Moreover, the technique used for freezing samples significantly affects CK and LDH activity (8–10) and freezing and thawing may adversely affect LDH isoenzymes (11–12).

As part of studies to characterize specific endocrinological variables in human prostatic cancer, we are examining CK and LDH activity and isoenzyme distribution in plasma and prostatic tissue. These evaluations would be facilitated if samples could be routinely stored before analysis. To assess this possibility critically, we examined the stability of CK and LDH activity and their isoenzyme distributions in our preparations. We report the results of these evaluations here.

Materials and Methods

Patients' specimens. All tissues were obtained from consenting patients at the time of diagnostic or palliative surgery for prostatic dysfunction. Specimens were placed in ice-cold Earle's medium (Grand Island Biological Co., Grand Island, NY 14072) after excision and were transported to the laboratory on ice. Tissues were blotted with filter paper, cut into pieces of approximately 125 mm³, packaged in individual heat-sealed packets (Kapak Corp., Bloomington, MN 55431), frozen between blocks of solid CO₂, and stored at −90 °C.

Blood was collected into heparinized tubes. Because the blood was to be used for a variety of analyses, each "plasma specimen" consisted of a mixture of equal volumes of three plasma samples obtained from three sequential bleedings performed at 20-min intervals. The blood was immediately cooled by immersing the container in ice, and plasma was rapidly separated by centrifuging the heparinized blood (2000 × g, 2 °C, 30 min). Plasma from the sequentially drawn blood samples was mixed, apportioned into glass vials, and stored at −90 °C. Plasma was thawed, analyzed, and then discarded.

Tissue fractionation. All procedures were performed in a refrigerated room at 2 to 4 °C. Frozen tissue was finely minced with a scalpel and homogenized with a glass–glass homogenizer in five volumes of "TES" buffer (50 mmol/L Tris, 0.1 mmol/L EDTA, 380 mmol/L sucrose; pH 7.4). A portion of the homogenate was removed and the remainder was centrifuged (5 min, 50 000 × g). The supernatant fluid constituted the clarified tissue extract. Aliquots of the homogenate and tissue extract were used for characterization of CK and LDH activity and isoenzyme content. Additional aliquots were stored at −90 °C in glass capillary tubes or glass vials for use in subsequent analyses.

Quantification of enzyme activity. CK activity was quantified in either plasma or tissue cytosols by use of a coupled enzymatic assay based on the method of Kim et al. (13). The incubation buffer (pH 6.8) contained, per liter, 100 mmol of imidazole, 1.0 mmol of ADP, 5.0 mmol of AMP, 20 mmol of p-glucose, 10 mmol of magnesium chloride, and 10 mmol of diithiothreitol. The incubation medium was stored at −20 °C for as long as 45 days. Cofactor, 20 mmol/L NADP⁺, and substrate, 200 mmol/L creatine phosphate, were prepared in incubation buffer immediately before use. Coupling enzymes, hexokinase (EC 2.7.1.1) and glucose-6-phosphate dehydrogenase (EC 1.1.1.49), were prepared in water at 200 and 100 U/L, respectively, and stored at 2 °C. The enzyme mixture was diluted 10-fold into incubation buffer just before use. Enzyme activity was assayed by adding plasma (approximately 30 µL) or tissue cytosol (10 or 20 µL; 100-fold dilution) to incubation buffer to give a final volume of 350 µL. Cofactor (50 µL) and coupling enzymes (50 µL) were added with mixing to duplicate tubes. Reaction was initiated by adding 50 µL of substrate. The reaction was monitored by the change in absorbance at 340 nm as determined against a blank that received 50 µL of incubation buffer instead of substrate. Incubation was at 30 °C and absorbance changes were monitored at 50-s intervals for 12 to 20 min with a Beckman DU 8 spectrophotometer equipped with a kinetics module and thermostated cuvette carrier.

Lactate dehydrogenase activity was quantitated in either plasma or tissue extracts essentially according to the method of Buhl et al. (14). Incubation was in a final volume of 500 µL containing, per liter, 100 mmol of imidazole, 150 µmol of NADH, and 1.0 mmol of sodium pyruvate; the final pH was 7.0. Plasma samples accounted for less than 5% and tissue extracts were less than 0.5% of the volume of these incubation mixtures. Assays were initiated by adding 50 µL of substrate and were read against reaction blanks identically constituted but lacking substrate. Incubation was at 25 °C for 5 to 7 min and product formation was monitored by...
measuring the absorbance change at 340 nm at 50-s intervals with the Beckman DU 8.

Characterization of isoenzymes. CK isoenzymes were separated by electrophoresis on Super Sephraphore (Gelman Sciences, Ann Arbor, MI 48106) cellulose membranes. CK isoenzymes were made visible by incubation at 37 °C, with use of the Gelman CK UV reagent set. CK isoenzymes were quantitated by fluorescence spectrometry (long wavelength ultraviolet excitation) in a Clifford Instruments (Corning Medical, Medfield, MA 02052) Model 445 Densicomp densitometer.

LDH isoenzymes were separated by electrophoresis on agar gel in the Microzone apparatus (Beckman Instruments, Fullerton, CA 92634). The isoenzymes were made visible by reaction at 37 °C, with use of the Beckman lactate dehydrogenase colorimetric reagent kit and were quantitated in the Densicomp densitometer by scanning the gels at 600 nm.

Results

Definition of assay conditions. We found that homogenates of human prostates or clarified tissue extracts had to be diluted 100-fold before CK or LDH activity could be quantified. With these preparations, the rate of change in absorbance at 340 nm was constant for an incubation interval of 8 to 20 min. This response was usually obtained with 5 to 20 μL of diluted homogenate or clarified tissue extract for CK assays or 20 to 60 μL of diluted homogenate or clarified tissue extract for LDH assays. It corresponded to maximum changes of 0.02 and 0.015 absorbance unit per minute for CK and LDH assays, respectively. Comparable experience with plasma preparations required incubation of 2.5 to 10 μL in the case of LDH assay or 30 to 60 μL in the case of CK assay. Under these assay conditions, enzyme activity was linearly related to sample volume incubated.

Isoenzyme quantitation. Plasma LDH isoenzyme distribution was independent of sample dilution, which yielded families of nonparallel lines converging at a common intercept on the abscissa (Figure 1). Similar analyses of homogenates of prostatic tissue from three different patients showed that LDH isoenzyme distribution was not independent of sample dilution (Figure 2). The effect of dilution appeared to be preparation specific (Figure 2) and prevailed even when the activity of individual isoenzymes approached that characteristic of plasma (compare Figures 1 and 2).

These results suggest that relative LDH isoenzyme distribution also may be sensitive to dilution effects during tissue homogenization. We examined this possibility by preparing a homogeneous tissue mince and homogenizing identical weighed aliquots with increasing volumes of homogenization medium. Apparent LDH isoenzyme distribution was highly sensitive to tissue dilution during homogenization (Figure 3A). To determine whether this phenomenon represented a peculiarity of tissue homogenization in the chosen buffer or a more generalized effect of dilution, we sequentially diluted one preparation with increasing quantities of electrophoresis buffer just before applying it to the gel. We found (Figure 3B) the dilution phenomenon characteristic of homogenization was duplicated. The quantitative similarities of the two dilution protocols were striking (compare Figure 3, panels A and B). Subsequent analyses were performed according to a standardized protocol in which tissue was homogenized in five volumes of buffer and isoenzyme distribution was evaluated in undiluted homogenates.

The effects of dilution on CK isoenzyme distribution were not rigorously evaluated because the level of CK activity in nearly all samples obviated dilution. Examination of selected prostatic homogenates diluted sixfold did not provide any
indication that dilution altered the apparent CK isoenzyme distribution.

Effects of storage on CK and LDH activities and isoenzyme distribution. There was no significant effect of storage at 
\(-90\, ^\circ C\) on CK or LDH activity in plasma or prostatic homogenates. These preparations could be stored for up to six weeks at \(-90\, ^\circ C\) and thawed once to 2 to 4 °C without any significant loss of enzymatic activity (Table 1).

Storage of either plasma or prostatic homogenates at 
\(-90\, ^\circ C\) for 160 to 180 days had no significant effect on LDH isoenzyme distribution. This result did not appear to reflect some peculiarity of the specimens analyzed, because prostate samples from the four patients showed different isoenzyme distributions, which also were not similar to that characteristic of plasma (Figure 4). CK isoenzyme distribution in plasma or prostatic homogenates also was unaffected by storage at \(-90\, ^\circ C\) for as long as 160 days (Figure 5). Storage of plasma at 2 to 4 °C for 24 h had no effect on CK or LDH isoenzyme distribution when compared with either “fresh” or frozen plasma (Figures 4 and 5).

![Figure 3](image1.png)

**Figure 3.** Effect of buffer volume during homogenization or addition of electrophoresis buffer to homogenates on the distribution of LDH isoenzymes

![Figure 4](image2.png)

**Figure 4.** The effect of storage of prostatic homogenates or human plasma at either \(-90\, ^\circ C\) or 2 to 4 °C on LDH isoenzyme distribution

Table 1. Effect of Storage on Plasma or Prostatic CK and LDH Activity

<table>
<thead>
<tr>
<th>Patient</th>
<th>Storage interval, wk</th>
<th>Prostate, U/g wet wt</th>
<th>Plasma, U/L</th>
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<tbody>
<tr>
<td>Patient</td>
<td>0 1 2 4 6</td>
<td>0 1 2 4 6</td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>106 106 98 105 99</td>
<td>52 48 48 54 54</td>
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<tr>
<td>B</td>
<td>145 139 142 143</td>
<td>42 42 51 47 47</td>
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<tr>
<td>C</td>
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<td>37 30 33 37 40</td>
<td>53 53 55 52 54</td>
<td></td>
</tr>
<tr>
<td>E</td>
<td>94 113 98 105 125</td>
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**Table 1.** Effect of Storage on Plasma or Prostatic CK and LDH Activity

<table>
<thead>
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<th>Enzymatic activity</th>
<th>Prostate, U/g wet wt</th>
<th>Plasma, U/L</th>
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<tr>
<td><strong>Creatine kinase</strong></td>
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<td>A</td>
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<td>85 110 95 109 110</td>
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<tr>
<td>B</td>
<td>50 47 45 49</td>
<td>102 121 133 113 133</td>
</tr>
<tr>
<td>C</td>
<td>44 43 46</td>
<td>162 173 150 160 170</td>
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<tr>
<td>D</td>
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<td>E</td>
<td>94 113 98 105 125</td>
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</tbody>
</table>

**Table 1.** Effect of Storage on Plasma or Prostatic CK and LDH Activity

**Discussion**

We have found no evidence that storage of human plasma for up to six weeks at \(-90\, ^\circ C\) deleteriously affects total CK activity (Table 1) or isoenzyme distribution (Figure 5). Our results are consistent with the studies of Szasz et al. (2), who found endogenous MM isoenzyme to be stable in human
serum stored at −20 °C for as long as four weeks. Our results are not in accord with the studies of Nealon and Henderson (1) or Morin (8), who describe significant losses of MM and MB isoenzymes (i) or MB isoenzyme (8) during storage at −80 °C. The differing results may be a consequence of the fact that (a) our specimens were plasma rather than serum reconstituted with purified CK isoenzymes and (b) we thawed our specimens at 2 to 4 °C rather than 37 °C (1) or 38 °C (8). Thermal instabiliy of CK isoenzymes is well documented (1, 2, 5, 8, 15). Finally, our results for plasma CK would not appear to be the consequence of using specimens poor in BB isoenzyme, because we obtained comparable results for frozen samples of human prostatic tissue homogenates that contained BB as the principal isoenzyme (Figure 5).

Storage of human plasma for four to six weeks at −90 °C did not alter total LDH activity (Table 1) or isoenzyme distribution (Figure 4). These results are in accord with the observations of Michie et al. (9), who found no significant decrease in LDH activity in patients’ sera which were quick frozen (acetone–solid CO₂ bath, −70 °C) and stored for as long as 48 h at −2 to −4 °C. Our observations are not in agreement with the studies of Kreutzer and Fennis (6) or Amelung et al. (7), who described marked instability of total serum LDH activity during storage at −8 to −10 °C. It is perhaps notable that Kreutzer and Fennis (6) remarked that "sera that were stored at −10 °C appeared not to be frozen." These same investigators (6) also reported marked cryo-instability of LDH-4 and LDH-5 isoenzymes. We found no indication of LDH isoenzyme cryo-instability for specimens stored either at 2 to 4 °C (24 h) or at −90 °C (Figure 4).

Stability of either CK and LDH enzyme activity or isoenzyme distribution in frozen prostatic homogenates paralleled that of frozen plasma (Table 1, Figures 4 and 5). We were unable to resolve the unexpected observation that prostatic LDH isoenzyme distribution as determined by agarose gel electrophoresis was sensitive to sample dilution during preparation (Figure 2). This would not appear to be a general phenomenon of agarose gel separation of LDH isoenzymes, because it was not characteristic of plasma samples (Figure 1). Our experience emphasizes the importance of validating and standardizing analytical protocols in detail before they are used for quantitative analysis of new types of samples.

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References