Creatine Kinase Isoenzymes in Neonate Plasma by Cellulose Acetate Electrophoresis: Albumin and Adenylate Kinase Artifacts

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Patterns of creatine kinase (CK, EC 2.7.3.2) isoenzymes were studied in apparently healthy one- to 10-day-old neonates, by use of a sensitive fluorescent staining method with Sclavo CK-F/6001 reagent. Mean activities of CK₁ (MM, 105 U/L), CK₂ (MB, 6.8 U/L), CK₁ (BB, 11 U/L), adenylate kinase (EC 2.7.4.3) anodal to CK₁, and a fluorescent albumin artifact were found. Pooled plasma from neonates is recommended as a control because it defines the albumin artifact and approximates the activity of CK₂ that must be observed after proper staining before a diagnosis of myocardial infarction can be made.

Additional Keyphrases: myocardial infarction · reference interval · fluorescence staining · analytical error

Measurement of creatine kinase (EC 2.7.3.2; CK) isoenzymes in serum has played an increasingly important role in the diagnosis of myocardial infarction (MI) (1-4). However, these isoenzymes have not been extensively studied for neonates, presumably because MI is found almost exclusively in adults. The electrophoretic separation methods used for CK isoenzymes in neonates (5-9) do not describe contributions for adenylate kinase (EC 2.7.4.3; AK) and albumin as artifacts in the electrophoresis patterns for neonates; such artifacts, if present, can complicate the interpretation of patterns for adults for MI diagnosis (10-12).

In this report we describe the CK isoenzyme content of neonate plasma, corrected for AK and albumin artifacts by using a staining method on cellulose acetate with Sclavo CK-F/6001 reagent. We recommend that pooled neonate plasma obtained from routine bilirubin analysis be used as a control, to prevent confusion from artifacts in the estimation of CK₂. Furthermore, activities of CK₁ and CK₂ in neonate plasma are much lower than those found in commercially available control sera and thus resemble the lower range of CK₂ found in cases of suspected MI for adults.

Materials and Methods

Specimens: Neonate plasma was obtained from skin-puncture blood drawn by heelsticks into heparinized Caraway tubes for bilirubin determinations. Any remaining plasma was stored at -20 °C within 2 h after the blood was drawn. Serum from normal ambulatory adults, obtained from surgical outpatient and laboratory volunteers, was also stored at -20 °C.

Reagents: Titan III Iso-Flur cellulose acetate electrophoresis plates (6 X 7.5 cm), buffer, electrophoresis chamber, and sample applicator were obtained from Helena Labs., Beaumont, TX 77704. Fluorescent staining reagent for CK isoenzymes, based on the Meziattini et al. (11) modification of the Rosalki method (13), in which fluoride and AMP inhibit about 95% of the AK, was obtained from Sclavo, Wayne, NJ 07470. We dissolved the reagent in one vial (Sclavo CF-F/6001) in 2.4 mL of the accompanying buffer and added 0.6 g of sucrose. Total CK was analyzed at 30 °C with an automated spectrophotometer (Model 3500; Gilford Instruments Labs., Oberlin, OH 44074) and Statzyme CPK Reagent (14) from Worthington Diagnostics, Freehold, NJ 07728; this reagent also contains fluoride and AMP for inhibition of AK.

We also used staining reagents similar to the Sclavo CK-F/6001 formulation except for variations in the AK inhibitors, fluoride, AMP, and creatine phosphate. Imidazole, creatine phosphate, ADP, N-acetylcysteine, NADP⁺, magnesium acetate, hexokinase (EC 2.7.1.1, from yeast), and glucose-6-phosphate dehydrogenase (EC 1.1.1.49, from Leuconostoc mesenteroides) were obtained from Sigma Chemical Co., St. Louis, MO 63178. Sodium fluoride and D-glucose were from Mallincrodt, Inc., St. Louis, MO 63147.

Electrophoresis was performed according to the Helena method except that separation was continued for 20 min rather than 10 min. Fluorescent spots for CK isoenzymes were stained by the Helena method with the Sclavo reagent for 1 h.

Preparation of pooled neonate plasma as a routine control: When we had collected about 2 mL of pooled neonate plasma remaining after bilirubin determinations, we removed the tube of pooled plasma from storage at -20 °C and gently thawed it by holding it in our hands. The plasma was mixed and centrifuged, aliquoted into microhematocrit tubes, and plugged at each end with clay. The plugged tubes were stored again at -20 °C in a light-tight container. Single microhematocrit tubes were removed each day and used as the CK control.

Results

For all neonate plasma studied we found substantial activities of CK₁, CK₂, and CK₃ as well as the fluorescent albumin-bilirubin artifact, as shown in Figure 1. Furthermore, when we used the Sclavo CK-F/6001 reagent to stain for CK activity, 87% of the samples showed well-formed shoulders on the anodal side of the CK₃ peak.

We investigated the nature of the anodal shoulder on the CK₃ peak by staining for activity with and without creatine phosphate in the staining mixture with no inhibitors of AK, as shown in Figure 2A and 2B. Deleting the CK substrate does not diminish the shoulder but eliminates the CK₃, CK₂, and CK₁ peaks.

Addition of fluoride (2 mmol/L) and 5'-AMP (6 mmol/L) inhibited AK by about 85% (Figure 2C); increasing the fluoride to 60 mmol/L inhibited AK by >95%. These results were verified by using the same reagents and measuring total AK with a Micro Centrifugal Analyzer (Instrumentation Laboratory, Lexington, MA 02173) programmed for total CK.

Adding NaF to the Sclavo CK-F/6001 reagent, to a final concentration of 60 mmol/L, also completely eliminated the anodal CK₃ shoulder (data not shown), confirming the results in Figure 2.
Similar experiments with sera from patients with above-normal CK₂ activity showed no significant change in the CK₂ percent of total CK activity as the fluorode was increased. Studies with a serum specimen from a hospital patient with increased cathodal CK₃ also showed no change in the percent of cathodal CK₃ as the fluorode was increased.

To study the linearity of the staining method with Sclavo CK-F/6001 reagent, we made serial dilutions of a serum specimen containing high activities of CK₃ and CK₂ (data not shown). Results by the method are essentially linear with the CK₂ peak proportion of total CK, if the total CK is <300 U/L. Above 300 U/L there were "flat-tops" in the CK₃ peak, owing to substrate depletion, which resulted in falsely increased values for CK₂.

The mean, standard deviation, and 95% reference intervals for the CK isoenzymes for neonates and adults are shown in Table 1. We also saw a shoulder, or peak cathodal to CK₃, in 90% of the specimens from adults.

Routine use of pooled neonate plasma as a control marker for CK isoenzymes resulted in a day-to-day CV of 12.1% (n = 42). There was no detectable trend for the loss of any of the CK isoenzymes in the control for a three-month period.

We investigated the possibility that the AK activity found in neonate plasma might be an artifact of heelstick collection or of the presence of heparin. Blood was obtained by finger skin puncture and by venipuncture with and without heparin from three normal adults. Electrophoresis showed no AK in any of the samples, suggesting that the presence of heparin or the use of heelsticks does not cause an AK artifact. Simultaneous samples of blood obtained from neonates heelstick and venipuncture were not available to compare for collection artifacts.

**Table 1. CK Isoenzyme Activity (U/L) for One- to 10-Day-Old Neonates and for Normal Ambulatory Adults**

<table>
<thead>
<tr>
<th></th>
<th>Neonates (n = 44)</th>
<th>Adults (n = 45)</th>
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<tbody>
<tr>
<td></td>
<td>Mean (SD)</td>
<td>95% Interval</td>
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<tr>
<td>Cathodal CK₃</td>
<td>0 (0)</td>
<td>0</td>
</tr>
<tr>
<td>CK₃</td>
<td>105 (95)</td>
<td>18–357</td>
</tr>
<tr>
<td>CK₂</td>
<td>6.8 (5.9)</td>
<td>2–26</td>
</tr>
<tr>
<td>CK₁</td>
<td>11 (7.5)</td>
<td>3–30</td>
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<tr>
<td>Total CK</td>
<td>133 (112)</td>
<td>26–396</td>
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**Discussion**

The mean activities of CK₁ (11 U/L), CK₂ (6.8 U/L), and CK₃ (105 U/L) (30 °C) for one- to 10-day-old neonates appear to be somewhat lower than other reports. We observed two CK₂ values >800 U/L but did not include these in the final statistical calculations because they were judged to be greater than 3 SD above the mean. Jouppila et al. (5) found about 30 U/L for CK₂ and 7 U/L for CK₁ in one-day-old neonates after normal delivery; assay temperature was not indicated. Von Beyer et al. (6) also reported higher mean values at 30 °C: CK₁ 32 U/L, CK₂ 45 U/L, and CK₃ 823 U/L. Schmidt et al. (7) reported a mean of 15.7 U/L for CK₂ in serum of newborns, whereas Jung et al. (8) reported CK₁ values of 5 U/L (37 °C) for children younger than one year old. In contrast to our work, Becker and Menzel (9) found CK₁ only in newborns with perinatal brain damage. None of these studies mentioned to aliquots of CK₁ and CK₂ for neonates and adults.

Neonates are actively involved in the process of replacing the erythrocytes containing hemoglobin F with cells containing hemoglobin A. This hemolytic process normally leads to increased concentrations of bilirubin, a breakdown product.
of the heme group in hemoglobin. Because erythrocytes also contain high concentrations of AK, neonates should also demonstrate increases of this enzyme in plasma. The extent of inhibition of plasma AK by Sclavo CK-F/6001 (about 85%), which is less than the 95% claimed by Meiattini et al. (17), may be adequate for specimen from adults but appears to be inadequate for the AK in neonate plasma. Increasing the fluoride concentration in the Sclavo reagent to 60 mmol/L appears to give essentially complete AK inhibition without affecting the CK₂ proportion of total CK.

Pooled plasma from newborns after bilirubin determination is recommended as a valuable control for use in the identification of CK isoenzyme positions and the albumin artifact, and for quantitative analysis of CK₂ and CK₁ activity. We believe that the mean neonate activities of CK₂ (6.8 U/L) and CK₁ (11 U/L) are very appropriate for use as a control, being only slightly above the upper reference value, 2.2 U/L, for normal adults (Table 1) (14). Regardless of the method of electrophoresis or staining procedure for CK isoenzymes, poorly stained plates would not show the CK₁ and CK₂ spots for the neonate control. Therefore a repeat of the procedure or, more drastically, modifications of the entire method would be required to improve sensitivity. Thus, we believe that neonate plasma is a valuable control for eliminating possible confusion in the diagnosis of MI in cases where the patient's serum CK₂ activity is at or near the upper end of the normal reference interval.

References