Selinive, Rapid Assay of Subforms of Creatine Kinase MB in Plasma

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The subforms of creatine kinase (CK; EC 2.7.3.2) in plasma have received recent attention as potential markers for the early diagnosis of acute myocardial infarction. Because changes in CK-MM subforms are not specific for myocardial injury, we developed an assay, based on high-voltage electrophoresis, that is sufficiently sensitive to detect the CK-MB subforms at concentrations substantially below the upper limit of normal (14 U/L). The assay can detect 1.25 U of either MB subform per liter with a precision of 0.20 U/L and gives responses that vary linearly with activity concentration from 0.0 through 30.0 U/L, with an identical signal response for both subforms. When both subforms are present in a serum sample, the assay accurately measures both the relative percentage and the absolute quantity of each: assay activity/known activity was 1.03 for each subform at a total MB subform activity of 5.0 U/L ($r = 0.98$). Assay time is 25 min, and there is no loss of CK during electrophoresis. Thus, this system can be used to rapidly, sensitively, and precisely quantify the two CK-MB subforms at activities well within the normal reference interval.

Additional Keyphrases: isoenzymes, myocardial infarction, electrophoresis, high-voltage

Thrombolytic therapy has become standard care in the treatment of acute myocardial infarction (AMI). To be maximally effective, therapy must be implemented in the initial hours of infarction (1, 2), so the decision to treat cannot be based upon conventional biochemical markers of AMI, which may remain within the normal range for 6 h or more after onset of symptoms (3). Consequently, treatment, which carries some risk of hemorrhage (4), must be implemented on the basis of clinical and electrocardiographic data, which are of limited diagnostic accuracy in the early hours of AMI. Thus, there are continuing efforts to develop techniques that would more quickly provide a reliable diagnosis.

The subforms of creatine kinase (CK; EC 2.7.3.2) have received recent attention as potential markers for the early diagnosis of AMI and for assessing the success of thrombolytic therapy (5–7). Studies of the CK-MM subforms demonstrated a transient increase in the relative proportion of the tissue subform, MM3, in comparison with the plasma-modified subforms, several hours before the total CK activity exceeded the normal reference interval. A major concern in the routine use of the subforms of the MM isoenzyme as diagnostic markers of myocardial injury is lack of specificity: significantly increased concentrations of MM3 in plasma are associated with skeletal-muscle injury of diverse etiologies, including inflammatory disease and trauma associated with exercise (8–10). A similar analysis of subforms of the MB isoenzyme of CK theoretically would provide the necessary specificity, but until now has been prohibited by the lack of an assay that is sufficiently sensitive to detect changes in CK subform composition in blood at activity concentrations within the normal range for CK-MB (11).

Consequently, we have developed and validated a rapid electrophoretic assay for the CK-MB subforms in plasma that is accurate and precise for MB activity concentrations that fall well within the normal reference interval.

Materials and Methods

Preparation of MB subforms: We purified MB2 from human myocardium as previously described (12). We could completely convert MB2 to MB1 by incubating 200 μL of purified MB2, 14,000 U/L in a pH 7.4 buffer (per liter, 10 mmol of Tris HCl and 20 mmol of β-mercaptoethanol), with 20 μL of a 500 mg/L solution of carboxypeptidase-B (Sigma Chemical Co., St. Louis, MO) for 15 min at 37 °C (7). We then added ethylene glycol bis(β-aminoethyl ether)–N,N,N’,N’-tetraacetic acid (EGTA) to give a final concentration of 30 mmol/L. To reproduce clinical assay conditions, we made all dilutions of these high-activity stock subform solutions in human serum in which the CK had been inactivated by incubation for 24 h at 55 °C. CK-MB activity was determined by a kinetic batch-adsorption assay (13), performed in duplicate on stock solutions of purified CK-MB and on the subsequent dilutions made from these samples. The upper normal limit for CK-MB in our laboratory by this assay is 14 U/L. Values reported represent enzyme activity at 30 °C.

Subform assay: CK subform samples were assayed in a "Rep" high-voltage electrophoretic unit (Helena Laboratories, Beaumont, TX) modified to accommodate a downward-directed heating element to provide uniform gel drying. A 1.0-μL sample of serum was mechanically loaded in each lane of a thin-layer 12 g/L agarose gel having low electrophoresis characteristics. These gels were designed to generate less heat under subform assay conditions than do commercially available CK isoenzyme gels. Reference lanes contained samples with subform activity of 0.0 and 20.0 U/L. Electrophoresis was at 1400 V for 12 min to resolve the subform bands; the manufacturer’s recommended conditions for conventional isoenzyme assay are 1200 V for 2 min. During electrophoresis the gel was cooled from below by thermostatically controlled peltiers to prevent melting of the agarose or denaturation of CK. After electrophoresis, CK substrate (CK reagent, Helena Laboratories), containing reagents required for the coupled enzymatic Rosalki reaction (14), was applied to the gel by a computer-directed robot gantry, and the gel temperature was increased to 45 °C for 5 min to permit generation of NADPH in situ at the location of the MB subform bands. We then dried the gels at 60 °C for 8 min and measured.

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3 Nonstandard abbreviations: AMI, acute myocardial infarction; CK, creatine kinase; MM, MB, isoenzyme CK-3 and CK-2; and EGTA, ethylene glycol bis(β-aminoethyl ether)–N,N,N’,N’-tetraacetic acid.

Received March 10, 1989; accepted April 11, 1989.
band density by scanning densitometry of visible light emitted by samples exposed to ultraviolet radiation (366 nm), as recommended by the manufacturer. The results, in arbitrary integral units per peak over baseline, were converted to activity-concentration units (U/L) by normalization to a linear scale constructed from the reference lanes.

To determine whether a non-CK artifact was a source of assay signal, we incubated several gels with the standard CK reagent but with creatine phosphate omitted. Total assay time, including data analysis, was 25 min.

In a subset of experiments, we measured the temperature of the gel surface during electrophoresis with an infrared camera (TUM 3000; Hughes Aircraft, Carlsbad, CA) mounted on an open Rep electrophoresis chamber that had been covered with infrared-transparent Teflon tape (CHR Industries, New Haven, CT). Images were recorded on videotape and temperatures determined from an internal calibration provided by the manufacturer.

Accuracy and precision: We reconstituted heat-inactivated serum with MB2 to give a final concentration of 1.25 U/L and performed the gel assay as outlined above. The mean subform activity, standard deviation, and coefficient of variation were determined within and between gels. Similarly, we repeated these experiments with MB2 activity of 2.5, 5.0, 10.0, and 30.0 U/L, and also with these same concentrations of MB1.

Linearity: Reconstituted serum containing MB2 activity ranging from 0.0 to 30.0 U/L was applied to consecutive lanes of a gel. Subform activity as determined by the assay was plotted vs known subform activity, and linearity was assessed by linear-regression analysis. The experiment was repeated with MB1, in the same activity range.

Relative subform ratio: To determine whether the densitometric signal response accurately reproduces the known subform ratio when both subforms are present in a sample, MB2 and MB1 were mixed in relative concentrations ranging from 100% MB2/0% MB1 to 0% MB2/100% MB1, in stepwise 10% increments. To prevent conversion of MB2 by any diluted carboxypeptidase-B remaining in the converted MB1 sample after pooling of the two subforms, we kept the samples on ice and assayed them immediately after mixing; in addition, all samples contained EGTA, a known inhibitor of carboxypeptidase, at 30 mmol/L (15). These experiments were performed at two activity concentrations for total CK-MB (i.e., the sum of MB2 + MB1 activity): 5.0 and 30.0 U/L. We determined the relative ratios of MB2/(MB2 + MB1) and MB1/(MB2 + MB1) and compared these with the known ratios. In addition, we determined the activity concentration of each subform, independent of its relative percentage, as outlined above.

Loss of CK-MB activity during electrophoresis: CK-MB undergoes substantial loss of activity at temperatures in excess of 25 °C (16). To assess whether such loss occurred during high-voltage electrophoresis protected by dynamic cooling, we compared the integrated signal response for samples that had been subjected to electrophoresis with that for samples that had been deposited directly onto the same gel and assayed without electrophoresis, as follows. Samples containing MB2 or MB1 at 10 U/L were subjected to electrophoresis under standard assay conditions. After electrophoresis, identical samples were mechanically deposited on the gel surface at a site remote from the anticipated location of the electrophoresed samples and allowed to adsorb into the gel. Because the CK-MB band of the nonelectrophoresed sample was not displaced from the albumin-associated fluorescent band, we deposited at a third site an equal volume of heat-inactivated serum containing no CK activity, so we could subtract the background fluorescence of the nonelectrophoresed sample. Reagent incubation, gel drying, and densitometry were performed as described above.

Results
We performed 434 assays on 34 gels. Of these, 10 lanes (2.3%) had to be excluded because of deposition of artifact (dust) on the gel surface before scanning; no single gel contained more than one such lane. Each subform produced a single peak, and no artificial bands were detected.

Accuracy and precision: Table 1 summarizes the mean and CV data for MB2 and MB1. The within-run CV ranged from 16% at an activity of 1.25 U/L to 4% at 30 U/L; between-run CVs were 19% and 5%, respectively, at the same activities. There was no difference in assay accuracy or precision between MB2 and MB1.

Linearity: Both MB2 and MB1 displayed a linear response from 0.0 through 30.0 U/L (n = 104; eight gels). The relation of subform activity determined by the electrophoretic method (y) and by the batch adsorption method (x) did not differ significantly between MB2 and MB1: for MB2, y = 1.01x - 0.19 U/L, r = 0.99, SE = 0.16 U/L; for MB1, y = 0.96x + 0.27 U/L, r = 0.99, SE = 0.21 U/L.

Relative subform ratio in a single sample: When both subforms were mixed in a single serum sample, the MB2/(MB2 + MB1) ratio, as determined by the relative densitometric integral ratio, accurately reflected the known MB2/(MB2 + MB1) activity proportion (Figures 1 and 2a). The assay was equally reliable whether the MB2 + MB1 activity was 5.0 or 30.0 U/L. Furthermore, the absolute activity of either subform in the mixed samples could be determined from the integration value for the corresponding peak, by comparison with a control lane having a known subform activity (Figure 2b).

Gel temperature during electrophoresis: Real-time infrared photography showed that the temperature at the gel

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surface increased to 25°C within the first minute of operation, then remained constant for the duration of electrophoresis.

Preservation of CK activity during electrophoresis: Samples subjected to high-voltage electrophoresis with thermostatically controlled cooling of the gel exhibited no loss of activity vs nonelectrophoresed controls. Mean assay activity was 10.5 U/L for samples undergoing electrophoresis (n = 12); mean activity for nonelectrophoresed samples corrected for background was 9.5 U/L (n = 12). There was no difference in response between MB2 and MB1.

Discussion

Our system involving high-voltage electrophoresis accurately quantified the CK-MB subforms in serum at activity concentrations that were less than 20% of the upper normal value. Even at concentrations as low as 1.25 U/L, the assay was precise to ±0.20 U/L within runs, and 0.23 U/L between runs. An earlier system used in our laboratory had a sensitivity of approximately 100 U/L (7). Chromatofocusing column chromatography used for assay of CK-MM subforms (17) is 20-fold less sensitive than the system described here, and with our system, separation of the subforms, detection, and analysis require only 25 min.

Although MB2 and MB1 differ by a single amino acid, we did not assume that their specific activity, and hence their densitometric signal response, would be identical. Indeed, we have previously demonstrated that MM3 and MM1 are cleared from the blood at different rates in the dog (15). MM3 and MM1 also appear to differ antigenically (18). Furthermore, preliminary data from our laboratory suggest that MB1 is more thermolabile than MB2 at 37°C. Consequently, we measured the densitometric signal per unit activity separately for MB2 and MB1; the signal varied linearly with concentration from 0.0 through 30.0 U/L, with an identical slope for the two subforms. Most importantly, when we mixed the two subforms in known proportions, the assay correctly quantified both their absolute activity and relative percentage. We performed these experiments at CK-MB activity concentrations of both 5.0 and 30.0 U/L; concentrations resulting in the activity range likely to be present in plasma during the first hours of AMI. Thus, if purified CK-MB controls are used as internal standards, this assay might obviate the need for a conventional assay CK-MB in the early hours of AMI.

We are investigating non-human CK-MB as a potential control material for this assay, to facilitate reliable inter-institutional quantitative subform measurement.

Although CK-MB is thermolabile, high-voltage electrophoresis resulted in no loss of CK-MB activity. This is most likely attributable to the controlled cooling of the gel during electrophoresis. We could not test this hypothesis directly, because the agarose gels melt in the absence of active cooling. However, infrared photography revealed a gel surface temperature of 25°C. Both subforms are stable in vitro at 25°C during incubations of less than 1 h, so we think that gel cooling probably is important for the preservation of MB subform activity during electrophoresis.

The subforms of CK-MB provide a potential means for earlier diagnosis of AMI. At baseline, MB2—the subform present in tissue—is in equilibrium with the serum-modified MB1 subform. Abrupt release of additional MB2 from tissue, as occurs in the initial hours of AMI, produces an upward shift in the MB2/MB1 ratio before CK-MB activity exceeds the normal range (19), similar to that shown previously for the subforms of CK-MM. However, because CK-MM is abundant in skeletal muscle, this ratio lacks the necessary specificity, analogous to the well-documented difference in diagnostic accuracy between conventional total CK assays and assays of the MB isoenzyme (20). Accordingly, assay of the subforms of the MB isoenzyme should provide similar specificity. A clinical study to evaluate the diagnostic sensitivity, specificity, and predictive value of CK-MB subforms is currently under way at our laboratory.

This study was supported in part by grant no. 3011 from the Texas Advanced Technologies Program, grant no. HL 36277 from the National Heart, Lung, and Blood Institute, NIH, and by the AHA/Physician Foundation Center for Molecular Biology in the Cardiovascular System.
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