may qualify to become a CDC-certified laboratory. In principle, because all laboratories are using the same instrument/reagent system, any matrix bias in frozen or lyophilized materials should be the same for all participants. Thus, the standardization laboratory can assign target values to frozen or lyophilized secondary standard materials for use by participating laboratories to assess their calibration. The principal limitation in such an approach will be the variable response to frozen or lyophilized materials owing to different lots of reagents and to inter-instrument hardware differences. Reagent-lot-specific target values for the secondary standards can be determined by having each independent lot of reagent tested by the standardization laboratory. The standardization laboratory can assay fresh serum samples and the secondary standards using its calibrated lot of reagents and the independent lot of reagents. The bias in fresh serum results between the calibrated and independent reagents will provide a lot-specific correction factor to apply to the apparent values obtained for the secondary standards with the independent lot of reagents. This system would be less complicated and less costly if the laboratories could all use the same lot of reagents with as long a shelf life as possible.

We thank the clinical chemistry technologists at the Medical College of Virginia for their assistance with donor sera collection, and we thank the technologists in all of our laboratories for their assistance.

References

CLIN. CHEM. 36/1, 149–153 (1990)

Mass Concentration and Activity Concentration of Creatine Kinase Isoenzyme MB Compared in Serum after Acute Myocardial Infarction

Joris R. Delanghe, Annick M. De Mo, Marc L. De Buyzere, Ivan K. De Scheerder, and Roger J. Wieme

We compared three current methods (immunoinhibition, "isomune-CK" immunoprecipitation, and the Tandem-E CKMB II immunoenzymometric assay) for determination of creatine kinase (CK; EC 2.7.3.2) isoenzyme MB in serum. Although results inter-correlated well, the immunoinhibition assay gave higher activity values. Atypical CK forms did not interfere with the immunoprecipitation and immunoenzymometric methods. In acute myocardial infarction the catalytic properties of CK decreased with the enzyme's age, as reflected by a steady increase in activation energy of the catalyzed reaction. In septicemia patients with very low CK and CK-MB catalytic activity, mean CK-MB mass concentration exceeded the upper reference limit, suggesting an increased rate of loss of activity concentration in these patients' sera. Because of the assay's lesser susceptibility to conformational changes at the active site of the enzyme, we suggest that measurement of CK-MB mass concentration is better suited for infarct sizing than measurement of catalytic activity.

Additional Keyphrases: enzyme activity · activation energy · septicemia · carboxypeptidase N · assessing infarct size

Measurements of creatine kinase (CK; EC 2.7.3.2) isoenzyme MB (CK-MB) concentration are widely used in the diagnosis and sizing of acute myocardial infarction (AMI).1

1 Nonstandard abbreviations: AMI, acute myocardial infarction; CK, creatine kinase; $E_A$, activation energy.

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Department of Clinical Chemistry (2B2), University Hospital, De Pintelaan 185, B-9000 Gent, Belgium.
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In the emergency laboratory, immunoinhibition assays for CK-MB are widely used, being technically simple and ideally suited for automation (1). However, the presence of atypical CK-forms and adenylate kinase (EC 2.7.4.3) in serum often leads to erroneous CK-MB results by various methods such as ion-exchange chromatography and immunoinhibition (2, 3). Corrections for the presence of these atypical forms presumably can be made by using a second precipitating antibody in the immunoprecipitation assays (4). Infarction sizes are frequently calculated from sequential measurements of CK-MB activities, but such data should be viewed with caution because of loss of specific activity of the enzyme after its release from the infarcted zone (5, 6). In addition, after effective thrombolytic therapy, calculations of activity-based infarct sizes are overestimated (7), partly because of these losses of specific activity.

Infectious intensive-care patients frequently demonstrate increased catabolism of CK in serum (8), attributable to the presence in their sera of a factor able to increase the activation energy (E_A) values of the catalyzed CK reaction. In these patients, mass-concentration measurements can be expected to give higher values than activity-concentration measurements. In cases where such factors are present in the patients’ serum in the early phase of an AMI, CK and CK-MB enzymatic activities may be unexpectedly low (9).

Here we compare mass and activity concentrations of CK-MB in serum, making use of commercial assays. Then we examine the relationship between mass and activity CK concentration as a function of time after the enzyme is released into the circulation. We also have evaluated whether CK-BB and macro-CK 1, the latter frequently observed in serum of patients with cardiac diseases (9), interfered with the mass CK-MB determinations. Finally, to understand better the biochemical nature of the low-CK phenomenon in intensive-care patients, we compared mass and activity concentrations of CK-MB in this population of patients.

Materials and Methods

Procedures

CK-MB catalytic activity. We measured total CK activity at 37 °C (10) in a "GEMSAEC" centrifugal analyzer (Electronucleonics, Caldwell, NJ 07006), using CK-NAC reagents (no. 3175; J. T. Baker, Deventer, The Netherlands). Catalytic CK-MB activity was measured after incubating the serum samples with CK-M subunit-inhibiting antibodies (from Isoimune-CK™; Roche Diagnostic Systems, Nutley, NJ 07110). Immunoprecipitation was also carried out by adding a second precipitating anti-goat IgG antibody (Roche Diagnostic Systems), both according to the manufacturer’s instructions.

CK-MB mass-concentration measurement. CK-MB mass concentration was measured with the Tandem-E CKMB II immunoenzymometric assay (Hybritech Inc., San Diego, CA 92121), according to the package-insert protocol. In this assay, serum samples are simultaneously incubated with CK-B-specific antibodies immobilized on a plastic bead and with CK-M monoclonal antibodies conjugated with alkaline phosphatase (EC 3.1.3.1).

Specific CK-MB activity. Specific CK-MB activity was calculated by dividing the value for catalytic CK-MB activity obtained with the immunoprecipitation assay by the value for mass concentration as measured with the Hybritech CKMB II assay.

Atypical CK-forms. The presence of atypical CK-forms was confirmed by use of a "Paragon" electrophoresis system (Beckman Instruments Inc., Brea, CA 92621). To study the effect of atypical CK-forms on CK-MB mass concentrations, we used three serum samples with normal CK-MB mass concentration (<9 μg/L) and containing high activities of macro-CK 1 (27 U/L, 60 U/L, and 133 U/L). Effects of CK-BB on CK-MB mass concentrations were tested by using a dilution series of human CK-BB standards (no. 749864; Scripps Laboratories, San Diego, CA 92121) with concentrations up to 500 U/L, in 50-U/L steps. To detect macro-CK 1 bound to the Hybritech beads, we used alkaline-phosphatase-labeled goat anti-human IgG antibodies (no. A-3150; Sigma Chemical Co., St. Louis, MO 63178).

After incubating 100 μL of serum and 100 μL of the Zero Diluent (provided in the Tandem-E CKMB II kit) with the bead in the absence of anti-M subunit antibodies at room temperature for 60 min, we incubated again for 90 min with 200 μL of goat antibody to IgG (dilution 1:1000). The presence of IgG-bound CK-BB was then elicited by incubating the bead in 200 μL of alkaline phosphatase reagent from the Tandem-E CKMB II kit during 30 min at room temperature, according to the manufacturer’s protocol.

CK-BB. Binding of CK-BB to the Hybritech beads was evaluated by incubating 200 μL of CK-BB standard solution (diluted similarly in Zero Diluent) with the bead at room temperature for 60 min. After three washing cycles with 2-mL portions of Wash Solution (from the Tandem-E CKMB II kit), the bead was incubated at room temperature with 200 μL of anti-B subunit antibody solution (originating from the former Hybritech Tandem CKMB kit) for 60 min. Finally, bound alkaline phosphatase activity was detected by a procedure similar to that used in the Tandem-E CKMB II kit.

Activation energy. Activation energy of the reaction catalyzed by CK was measured according to the Arrhenius equation: \( \ln k_1/k_2 = E_A/R (1/T_1 - 1/T_2) \) (5), where \( k_1 \) and \( k_2 \) represent catalytic activities measured at absolute temperatures \( T_1 \) and \( T_2 \) and \( R \) is the universal gas constant (8.314 J · mol⁻¹ · K⁻¹). The standard temperature interval for determination of the apparent activation energy was 25–37 °C. To measure catalytic activity at 25 °C and 37 °C, we used a GEMSAEC centrifugal analyzer. The changes in pH of the reaction mixture caused by use of the different temperatures were too small to necessitate correction factors.

Post-transcriptional CK-MB modification. Serum samples were incubated for 6 h at 37 °C with aryleuclafate (20 kU/L; EC 3.1.6.1, from Patella vulgata) and with sialidase (20 U/L; EC 3.2.1.18, from Clostridium perfringens), both from Sigma Chemical Co.

Patients

For this study we analyzed 49 serum samples from 21 patients admitted to our hospital because of AMI [16 men; mean age 65.9 (SD 9.5) y; five women, mean age 73.8 (SD 7.2) y]. Blood was sampled during the first three days after onset of symptoms. Twenty ostensibly healthy blood donors (mean age 42.7, SD 11.5 y) served as a control group. Ten patients (seven men, three women; mean age 64.8, SD 8.7 y) with macro-CK 1 (39 ± 28 U/L), seven suffering from unstable angina pectoris and three with a recent (one month prior to sampling) history of AMI were monitored, as were 10 patients (five men, five women; mean age 64.5, SD 6.1 y) being treated for septicemia in the Intensive Care Unit, who had very low CK (<10 U/L) activities. None of
these latter groups had clinical evidence of skeletal or heart muscle injury. We also studied one patient with a scintigraphically proven AMI but negative enzyme kinetics (maximum CK activity 11 U/L, CK-MB 0 U/L).

**Results**

**Precision Data**

The within-run CV for the three investigated methods was evaluated by use of a low- and a high-concentration pooled serum (Table 1). For the low-concentration sample CVs ranged from 3.0% to 3.7%, whereas for the high-concentration sample the imprecision of the immunoenzymometric assay was clearly higher than for the others.

**CK-MB Assays Compared**

Mass concentration measurements yield a good correlation with both immunoinhibition and immunoprecipitation techniques (Figure 1). Regression coefficients between the methods were similar: $r = 0.935$ for immunoenzymometric vs immunoinhibition assay and $r = 0.943$ for immunoenzymometric vs immunoprecipitation assay. Striking differences are observed for the values of the intercept: an extrapolated mass concentration of 0 µg/L corresponds, respectively, to 1.1 U/L for immunoprecipitation and 9.0 U/L for immunoinhibition. As a consequence, immunoinhibition gives significantly higher extrapolated background values for CK-MB than does immunoprecipitation.

**Interference of Atypical CK-forms**

In the 10-patient group with atypical CK-forms, immunoinhibition is interfered with by atypical CK-forms, but both the immunoprecipitation and the immunoenzymometric assay were unaffected by the presence of atypical CK. Multivariate analysis showed that $y$ (Tandem-E CKMB II, µg/L) = 1.58x (Isomune-CK, U/L) + 0.06 (atypical CK activity, U/L) − 3.94. The specific activity of CK-MB in these 10 patients was 0.62 (SD 0.16) U/µg.

**Binding of Macro-CK 1 and CK-BB to Anti-B Subunit Monoclonal Antibodies**

By making use of alkaline phosphatase-labeled antibodies directed against human IgG, we could demonstrate that macro-CK 1 binds to the anti-B subunit coated beads. Absorbance measured at 405 nm correlated with the amount of macro-CK 1 present in the three serum samples we studied. However, these atypical forms were not recognized by the second enzyme-labeled antibodies of the Tandem-E CKMB II assay. Similarly, CK-BB was shown to bind on the beads in amounts linearly related to concentrations of 350 µg/L, but was not recognized as well by the conjugated anti-M subunit antibodies of the Tandem-E CKMB II kit.

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**Table 1. Precision Study of Three Current Methods for Determination of CK-MB**

<table>
<thead>
<tr>
<th>Method</th>
<th>Low concentration</th>
<th>High concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>µg/L</td>
<td>µg/L</td>
</tr>
<tr>
<td></td>
<td>$\bar{x}$, CV, %</td>
<td>$\bar{x}$, CV, %</td>
</tr>
<tr>
<td>Immunoenzymometric</td>
<td>15, 3.7</td>
<td>44, 2.1</td>
</tr>
<tr>
<td>Immunoinhibition</td>
<td>30, 3.0</td>
<td>64, 1.0</td>
</tr>
<tr>
<td>Immunoprecipitation</td>
<td>18, 3.3</td>
<td>51, 1.1</td>
</tr>
</tbody>
</table>

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**Fig. 1. Correlation between serum CK-MB results obtained by (top) an immunoenzymometric assay and an immunoinhibition assay and (bottom) an immunoenzymometric assay and an immunoprecipitation assay**

The equation for the linear correlations are as follows: (top) $y = 1.40x - 12.39$ ($r = 0.935$, $n = 49$, $S_y = 21.21$) and (bottom) $y = 1.59x - 1.69$ ($r = 0.943$, $n = 48$, $S_y = 19.84$)

**Effect of Enzyme Aging**

When the specific activity of total CK was plotted vs time after onset of AMI symptoms, specific activity was seen to decrease with enzyme aging. The specific activity of serum CK-MB decreased from 0.88 (SD 0.40) U/µg during the first 12 h after onset to 0.71 (SD 0.30) U/µg ($P < 0.01$) by the third day after onset. Also the $E_A$ value of the CK reaction correlated qualitatively with loss of specific activity. Additional incubation of AMI serum with either sialidase or sulfatase for 6 h at 37 °C did not significantly change CK-MB mass measurements. Table 2 summarizes changes of specific activity of CK-MB and $E_A$ of the CK reaction as a function of time after onset of acute myocardial infarction.

**Low-CK Patients**

In 10 patients from the Intensive Care Unit with low CK activity (CK <10 U/L), CK-MB catalytic activity as determined by immunoinhibition (mean: 1 U/L; range: 0–4 U/L; reference values: 14.5 ± 4.5 U/L) and immunoprecipitation (<1 U/L; reference values: 2.6 ± 2.8 U/L) was very low. In these patients, the mean CK-MB mass concentration was 2.27 µg/L (range: 0.31–4.0 µg/L), significantly higher than
Table 2. Changes in Specific Activity of CK-MB and Its Activation Energy in the Course of AMI

<table>
<thead>
<tr>
<th>Time*</th>
<th>n</th>
<th>Specific activity, a</th>
<th>Activation energy, b</th>
</tr>
</thead>
<tbody>
<tr>
<td>4–12</td>
<td>13</td>
<td>0.88 ± 0.40 c</td>
<td>53.4 ± 2.8</td>
</tr>
<tr>
<td>12–24</td>
<td>12</td>
<td>0.82 ± 0.20</td>
<td>60.0 ± 2.9</td>
</tr>
<tr>
<td>24–48</td>
<td>12</td>
<td>0.77 ± 0.27</td>
<td>63.3 ± 3.0</td>
</tr>
<tr>
<td>48–72</td>
<td>12</td>
<td>0.71 ± 0.30 d</td>
<td>66.5 ± 3.0</td>
</tr>
</tbody>
</table>

* Hours after onset of retrosternal pain. a Measured at 37 °C. b Mean ± SD.

for the reference population (mean: 1.1 µg/L; range: 0.8–4.2 µg/L). In a patient with proven AMI who did not show any increase in serum CK and CK-MB activity in the post-infarction period, the mass concentration of CK-MB ranged between 0.6 and 3.1 µg/L. To study the effect of CK-deactivating factors present in low CK serum on the binding with the monoclonal antibodies of the Tandem-E CKMB II assay, we added serum from patients with very low CK activity to equal amounts of serum from an AMI patient (CK-MB concentration 120 µg/L). After the sample was incubated for 3 h at 37 °C, no significant differences in assay results were noticed, suggesting that these CK-deactivating factors are not directly involved in the binding site for the studied monoclonal antibodies.

Discussion

Statistical analysis of the three current CK-MB methods showed a good correlation between mass concentration and catalytic activity, but absolute values for CK-MB obtained by the different methods varied widely. For an equal amount of mass concentration, the immunoinhibition method gave higher CK-MB activities than did the immunoprecipitation technique, both for the low and high concentration ranges. In the low concentration range, differences in CK-MB results may originate from the presence of atypical CK-forms and adenylate kinase in serum. In this study, immunoprecipitation and immunoenzymometric assay techniques were shown not to be interfered with by macro-CK 1. Because the activity of adenylate kinase in serum is usually low (11), as are also the activities of atypical CK-forms (2), differences between the methods are more pronounced in the lower concentration ranges.

Variance between mass and catalytic CK-MB concentration can partly be ascribed to changes in enzyme structure, which are caused by carboxypeptidase N (EC 3.4.17.3) activity (6, 12). However, activities of the latter enzyme in serum are characterized by a large inter-individual variation (13). Because of the conformational changes in the CK-MM molecule, polyclonal antibodies against CK-M subunit fail to inhibit CK-MM1 activity completely in immunoinhibition assays. As a consequence, CK-MB activity is overestimated during the second and third day after infarction (14). Binding of CK-MB to the Hybritech Tandem-E monoclonal antibodies is not affected by carboxypeptidase N activity (15). Theoretically, differences in sialic acid and sulfate residues may also give rise to enzyme heterogeneity (16, 17). However, digestion with sialidase and arylsulfatase did not affect CK-MB mass-concentration measurements. Despite published data proving that CK is denatured in serum during the second and third day after infarction (6, 18), paradoxical increases of specific activity have been described during the decline phase of post-AMI CK-MB activity (19). Our results show a steady increase in CK-MB activation energy, which is consistent with the general concept of tissue-enzyme denaturation after release into the systemic circulation. However, the changes in specific activity are smaller than could have been expected from the data on activation energy, suggesting the induced conformational changes of CK-MB during its stay in the blood to be multifactorial. In comparison with the earlier Hybritech Tandem CKMB kit, specific CK-MB activity is higher (20).

In contrast to the immunoinhibition method, immunoprecipitation and immunoenzymometric assays are insensitive for macro-CK 1, consistent with earlier data (4, 21). Theoretically, extremely high concentrations of CK-BB and macro-CK 1 in serum samples can lead to saturation of the available binding sites for the B subunit on the coated beads and thus give rise to falsely negative results, but in almost all cases concentrations of CK-BB and macro-CK 1 are low (2, 22).

Owing to changes in activation energy on enzyme aging, the correlation between mass concentration and catalytic activity measured at 37 °C is more nearly linear than when measured at 25 °C. For infarct sizing, mass concentration measurements are therefore easier to interpret, because they better reflect the amount of CK-particles that have been released from the infarcted zone. Errors caused by conformational changes of CK on aging exceed the induced imprecision accompanying the use of immunoassays.

Despite the almost complete absence of catalytic CK-MB activity in low-CK patients, in some of them CK-MB mass concentrations are between the upper reference range and the cutoff value for AMI. Also, in the patient with proven AMI but no increase in the activity of CK and CK-MB in serum, discrepant results between catalytic activity and mass concentration of CK-MB were found. These results suggest that, in these patients' sera, specific CK-MB activity must be very high. This is consistent with earlier findings that, in these patients, CK catabolism is increased because of the presence of CK-deactivating factors (6). In patients with AMI and unexpectedly low CK and CK-MB activities (8, 9), we therefore recommend CK-MB to be remeasured by means of an immunoassay.

We thank Ma. Corinne Fordeyn for typing the manuscript.

References

Monoclonal Antibody Inhibiting Creatine Kinase MM₃ but Not Isoform MM₁

Tadeto Suzuki,¹ Takanari Shirahashi,¹ Kosuke Tomita,¹ Masayuki Totani,² and Takashi Murachi²

Monoclonal antibody CKM-G01 inhibited >99% of the activity of porcine and human creatine kinase MM-MM isoenzyme purified from muscle. However, it inhibited only 54% of CK-MM in human serum. Chromatofocusing of serum CK-MM showed that CKM-G01 inhibited 100% of MM₃ but not isoform MM₁. CKM-G01 inhibited CK-MM₂ by 57%. CKM-G01 specifically inhibited only the original CK-MM subunit and not the subunit modified by removal of C-terminal lysine by carboxypeptidase N. CKM-G01 can be used for assay of CK isoforms. We devised a new diagnostic reagent involving it, which requires no analytical separation of isoforms, based on the immunoinhibition method, and applied it to early diagnosis of acute myocardial infarction. The "inhibition index," (inhibited CK activity/total CK activity) × 100, increased more rapidly than did total CK and CK-MB. Evidently this diagnostic reagent can be used for easy, early diagnosis of acute myocardial infarction.

Additional Keyphrases: diagnosis of acute myocardial infarction · isoenzyme inhibition index · heart disease

Measurement of creatine kinase (CK; EC 2.7.3.2), especially the CK-MM isoenzyme, in serum is routinely used as a reliable biochemical method for diagnosis of acute myocardial infarction (AMI). There are several post-translationa isoforms (subtypes) of CK-MM and CK-MB isoenzymes (1). This diversity is caused by the action of carboxypeptidase N (CP-N) (2–4). The CK-MM originally present in cardiac muscle is the unmodified "tissue type," known as MM₃. Once MM₃ is released into plasma, CP-N sequentially converts the MM₃ to MM₂ by cleaving one of two C-terminal lysines of subunits, and then MM₁ is produced by cleavage of another C-terminal lysine (2).

Several recent papers have shown that individual CK-MM isoforms can be separately determined to allow calculation of the ratio of MM₃ to MM₁, which is a good index for earlier diagnosis of AMI (5, 6), early detection of reperfusion (7–9), and presumption of infarction size or onset time (10–12). Each isoform currently is determined by electrophoresis (1, 7), isoelectric focusing (5, 9), chromatofocusing (2, 10), or liquid chromatography (13)—methods that are difficult to automate and thus inadequate for routine laboratories. Therefore, a faster, easier method for determining the CK-MM isoforms is needed.

In the course of our studies on CK-MM-inhibiting monoclonal antibodies (14), we found one, designated CKM-G01, that inhibited CK-MM₃ by almost 100%, but not CK-MM₁. Here, we describe the inhibitory specificity of CKM-G01 for CK-MM isoforms and its potential usefulness for determination of the isoforms ratio in early diagnosis of AMI.

¹ Biochemistry Department, Research and Development Center, Unitika, Ltd., 29 Konakura, Uji, Kyoto 611, Japan.
² Department of Clinical Science and Laboratory Medicine, Kyoto University Faculty of Medicine, Sekyo-ku, Kyoto 606, Japan.


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