Analytical Evaluation of a Sensitive Enzyme Immunoassay for Determinations of Creatine Kinase Isoenzyme MB

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We have evaluated a new sensitive immunometric assay for the determination of creatine kinase (CK; EC 2.7.3.2) MB isoenzyme (NovoClone™ CK-MB), involving an enzyme label and two monoclonal antibodies directed against the B subunit and the M subunit, respectively. The anti-CK-B antibodies are bound to the solid phase. The assay was modified to be extremely sensitive and thus to measure the concentration range below and close to the cutoff value used for the diagnosis of myocardial infarction. A reference interval of 0–6 μg/L was found for 315 outpatients without myocardial diseases (132 men and 183 women); the overall median of the log-gaussian distribution was 1.91 μg/L (2.03 and 1.79 μg/L for men and women, respectively). Total and within-assay imprecision (CV) was <6% at the upper reference limit. The detection limit was 0.1 μg/L. The assay provides a favorable signal-to-noise ratio: the calibrators 0.0, 2.0, and 30.0 μg/L give absorbances at 492 nm of 0.040, 0.140, and 1.600, respectively. We conclude that the assay provides biochemical identification of individuals with myocardial damage but without myocardial infarction.

Determination of creatine kinase (CK; EC 2.7.3.2) MB isoenzyme (CK-MB) has been extensively used as a diagnostic test in myocardial infarction (1).4 Electrophoresis was the first available technique (2), but recently immunological methods have replaced electrophoresis in many laboratories. Some of the immunoassays are still not specific for CK-MB; for example, the B-subunit-specific radioimmunoassay (3) and the immuno-inhibition assay inhibiting the M-subunit (4) measure both CK-MB and CK-BB.

The analytical and clinical performance of these methods are not always satisfactory (5).

Direct measurement of CK-MB requires the use of both M- and B-specific antibodies, such as immunoradiometric (6) or immunoenzymometric assays (7), which measure mass concentration of the enzyme instead of its enzymatic activity. In these methods the anti-CK-M antibodies are bound to the solid phase. Most recently two immunoassays have been introduced that directly measure creatine kinase MB with monoclonal anti-CK-MB antibodies (8).

Here we report an evaluation of a new sensitive double monoclonal immunometric assay, NovoClone™ CK-MB, high-sensitivity version (Novo Biolabs, Cambridge, U.K.). The anti-CK-B antibodies are bound to the solid phase, and peroxidase-labeled anti-CK-M antibodies are used as detecting antibodies. The aim of the assay is to measure values found in individuals with myocardial damage but without acute myocardial infarction (AMI).

Materials and Methods

Materials. Kits for NovoClone CK-MB, high-sensitivity version, were supplied by Novo Biolabs. Each kit contains reagents sufficient for 96 determinations. Peroxidase-labeled anti-CK antibody, calibrators (0, 2, 5, 10, and 30 μg/L), and bovine serum used as zero-concentration serum were supplied lyophilized and were reconstituted according to the manufacturer's instructions. The reagents were stable for two weeks at 4 °C after reconstitution.

Proceedures. The assay procedure is as follows: pipet 100 μL of detecting antibody solution (peroxidase-labeled CK-M antibodies, 4 mg/L, in 50 mmol/L 4-(2-hydroxyethyl)-1-piperazineethanesulfonate buffer, pH 7.2, containing 0.5 mol of ammonium sulfate per liter) and 20 μL of serum (specimen, calibrator, or control) into each of the wells. Incubate the microtitration plate for 1 h at room temperature (20–25 °C) on an agitation board. The wells are washed five times with washing buffer. After adding 100 μL of o-phenylenediamine substrate solution (0.8 g/L in 0.1 mol/L citrate–perborate buffer, pH 5.2), incubate the microtitration plate for 15 min at room temperature on an agitation board. Stop the reaction by adding 100 μL of sulfuric acid (1 mol/L). Read absorbances at 492 nm with a
microtiter plate reader (we used a Vmax™ Kinetic Microplate Reader; Molecular Devices, Palo Alto, CA). Calculate the results by comparison with a linear calibration curve (four-point logistic curve fit).

Serum samples. For all experiments we used human serum or pools of human serum. To estimate assay imprecision, we analyzed four human serum pools and two lyophilized materials: Seronorm Enzyme, SN 803 (Nycomed A/S, Oslo, Norway), a bovine serum with added CK-MB, and HK 87, a lyophilized human serum produced for the Danish Society of Clinical Chemistry by Nycomed A/S. The six materials for the imprecision study were stored in small aliquots at −20 °C during the experiments.

For the estimation of the detection limit, a bovine serum containing zero CK-MB was used.

Patients' specimens. For the estimation of reference intervals we sampled blood from 315 outpatients (132 men and 183 women) without myocardial diseases. Serum was separated from blood cells within 1 h after venipuncture and analyzed the same day. The median age of the 132 men was 56 years (range 16–84 years). For the 183 women the corresponding median age was 58 years (range 14–95 years).

Further we investigated four patients without ischemic heart disease (non-IHD), 10 patients with stable angina pectoris (SAP), and 13 patients with unstable angina pectoris (UAP). The SAP group was characterized by typical attacks of chest pain provoked by physical exertion or psychological factors and pain relief by nitroglycerine. The diagnosis of UAP was based on chest pain at rest or brought about by minimal exertion, and (or) an entirely new pattern of chest pain in patients previously classified as suffering from chronic angina (9). None of the patients in the two groups had signs of myocardial infarction. Residual enzyme activity of CK-B measured after immunoinhibition of CK-M was for all patients within the normal range [<12 U/L (37 °C)] throughout the period of measurement.

On admission to hospital, patients with UAP were essentially managed identically in a general coronary care regime, including bed rest and control of cardiac pain by nitrates, morphine, and oxygenation. No patient suffered from hemodynamic disturbances or arrhythmias demanding medical treatment. During the period of blood sampling, all patients in the UAP group started aspirin and diltiazem treatment; four of these patients were given intravenous nitroglycerin (up to 200 mL/24 h). Therapy did not influence the interpretation of the analytical results, as evidenced by serum albumin remaining constant in each patient within the observed period.

The non-IHD and the SAP patients were exercised on bicycles—the non-IHD patients until exhaustion and the SAP patients until both pain and ST-depression of at least 0.1 mV occurred. Blood samples were drawn before and every 5–6 h to 24 h after exercise. Blood samples from the UAP patients were drawn on admission and every 3–6 h during the first 24 h and then every 8 h up to 72 h. Samples were drawn into dry glass containers through an in-dwelling catheter in an antecubital vein. After separation the serum was stored at −80 °C until analyses. Informed consent was obtained from each patient and the study was approved by the Regional Scientific Ethical Committee.

Statistical methods. We used the F-test to assess the significance between the variances, and Fisher's exact test for the significance of association between enzyme fluctuation and electrocardiogram/Holter analysis. At P ≤ 0.05, the difference was considered significant.

Results

Imprecision. Total and within-assay imprecision of the CK-MB immunoassay were estimated according to guidelines from the National Committee for Clinical Laboratory Standards (10). Four human serum pools and two reconstituted materials were analyzed twice in each of two separate runs for 14 days. Table 1 indicates that the variance is mainly due to the intra-assay component.

Detection limit and sensitivity. The detection limit, calculated as the mean + 3 SD for 26 replicates of the zero CK-MB serum, was 0.1 μg/L. The high sensitivity of the assay is demonstrated by the log–log calibration curve. E.g., calibrators for 0.0, 2.0, and 30.0 μg/L have absorbances of around 0.040, 0.140, and 1.600 A, respectively. This, together with a low imprecision, results in a high signal-to-noise ratio in the assay.

Linearity. We evaluated the linearity of the assay by analyzing samples mixed with known proportions of two serum specimens with low and high concentrations of CK-MB, respectively. The linearity was excellent within the range of the calibration curve (0–30 μg/L).

Specificity. The interference from matrix effects was investigated by diluting 11 different serum samples threefold with the zero CK-MB serum, thus diluting unspecific reactions. The mean values before and after dilution were 16.84 and 16.93 μg/L, respectively. No serum samples showed deviations between the results for diluted and undiluted samples.

Reference intervals. Reference intervals for serum CK-MB were estimated by using the 97.5% percentile as the upper reference limit (Figure 1). For both men and women, the upper reference limit was 6.0 μg/L. The distributions are log-gaussian, with medians of 2.03 and 1.79 μg/L for men and women, respectively.

Variability of CK-MB. Figure 2 shows the individual time courses of serum CK-MB concentrations as exemplified in three patients, one from the SAP group and two from the UAP group, with and without enzyme fluctuations. For comparison, the established discrimination limit of CK-MB for the diagnosis of AMI is ~25 μg/L. We calculated the variances of serum CK-MB concentrations in each patient during the first 24 h after exercise for the non-IHD and SAP groups and after the onset of chest pain in the UAP group. Because there was no difference in the variances of the individual patients in the non-IHD and SAP groups, we considered these as one group.

Table 2 compares the variances of serial determinations of serum CK-MB concentrations in each UAP patient with the pooled variance of the combined non-IHD/SAP group. Six patients with UAP had significantly greater serum

<table>
<thead>
<tr>
<th>Table 1. Within-Assay and Total Imprecision</th>
</tr>
</thead>
<tbody>
<tr>
<td>CK-MB, μg/L</td>
</tr>
<tr>
<td>Mean</td>
</tr>
<tr>
<td>HK-87</td>
</tr>
<tr>
<td>SN 803</td>
</tr>
<tr>
<td>Serum A</td>
</tr>
<tr>
<td>Serum B</td>
</tr>
<tr>
<td>Serum C</td>
</tr>
<tr>
<td>Serum D</td>
</tr>
<tr>
<td>n = 14 each.</td>
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</table>

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Table 2. Variability of CK-MB Concentrations in Serum
24 h after Onset of Chest Pain in Patients with
Unstable Angina Pectoris*

<table>
<thead>
<tr>
<th>Patient</th>
<th>Variance</th>
<th>n</th>
<th>Variance ratio</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.09</td>
<td>7</td>
<td>0.50</td>
<td>NS</td>
</tr>
<tr>
<td>2</td>
<td>1.22</td>
<td>6</td>
<td>6.78</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>3</td>
<td>8.63</td>
<td>6</td>
<td>47.94</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>4</td>
<td>1.29</td>
<td>6</td>
<td>7.17</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>5</td>
<td>0.02</td>
<td>4</td>
<td>0.11</td>
<td>NS</td>
</tr>
<tr>
<td>6</td>
<td>0.01</td>
<td>6</td>
<td>0.06</td>
<td>NS</td>
</tr>
<tr>
<td>7</td>
<td>0.08</td>
<td>7</td>
<td>0.44</td>
<td>NS</td>
</tr>
<tr>
<td>8</td>
<td>2.06</td>
<td>6</td>
<td>11.44</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>9</td>
<td>0.02</td>
<td>7</td>
<td>0.11</td>
<td>NS</td>
</tr>
<tr>
<td>10</td>
<td>0.01</td>
<td>7</td>
<td>0.06</td>
<td>NS</td>
</tr>
<tr>
<td>11</td>
<td>0.05</td>
<td>5</td>
<td>0.28</td>
<td>NS</td>
</tr>
<tr>
<td>12</td>
<td>0.86</td>
<td>7</td>
<td>3.78</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>13</td>
<td>0.74</td>
<td>7</td>
<td>4.11</td>
<td>&lt;0.05</td>
</tr>
</tbody>
</table>

*By comparison, the variance in 91 samples from non-IHD/SAP patients was 0.18.

n = number of samples. NS, not significant.

from that found in the combined group. Enzyme fluctuation was associated with electrocardiographic signs of subendocardial ischemia on admission ($P < 0.01$) and with signs of repetitive ischemic episodes deduced from continuous ST-segment monitoring during the first 24 h after admission ($P < 0.05$).

Discussion

Measurement of normal or slightly increased CK-MB concentrations in serum requires low imprecision of the assay, low detection limit, and a high signal-to-noise ratio. The NovoClone CK-MB kit has been designed to meet these demands.

With this assay we found the total CV to be about 6% within the reference interval; this is lower than has been reported earlier for CK-MB methods, where variations of at least 10% are seen (7, 8, 11, 12). The detection limit, calculated as the mean + 3 SD for 26 replicates of the zero CK-MB serum, was 0.1 µg/L. For comparison Chan et al. (11) reported a detection limit (+ 2 SD) of 3 µg/L. The excellent analytical performance of this assay is mainly attributable to the favorable signal-to-noise ratio.

The upper reference limit of 6 µg/L (97.5% percentile) for outpatients without myocardial disease is slightly higher than those reported by Chan et al. (11) and by Wu et al. (13) for 100 and 40 healthy individuals, respectively. On the other hand, it is in good agreement with the findings of Apple et al. (3) for 50 healthy persons. These differences probably reflect the different standardization of the assays and demonstrate the need for an approved standard preparation. Differences in the chosen populations may also be involved.

Wu et al. (13) demonstrated that measurement of mass concentration and of enzyme activity gives nearly identical results regarding the rate of CK-MB release from the heart, the time of the peak value in serum, and the rate of elimination of the isoenzyme from the circulation. This implies equivalence between the enzymatically active and immunologically reactive forms of CK-MB. The same is not true for quality-control materials, probably because the quality-control specimens contain a higher fraction of enzymatically inactive CK-MB than does fresh human serum (11). The immunoassays measure immunoreactive CK-MB,
which may be a combination of catalytically active and inactive enzymes. After the specimens had been stored at
20 °C, the immunochemical results were less affected
than the enzyme activity (14).

There are several perspectives in the application of this
sensitive assay. It may now be possible to detect minor
myocardial damage by evaluating results that are below
the present decision limit used for myocardial infarction. In
an earlier study of comparable design, Thygesen et al. (15)
showed that the variability of the non-IHD and the SAP
group were identical for both CK and CK-B after inhibition
of CK-M subunit activity. The UAP group showed variability
of CK between that of the AMI group and the non-IHD/
SAP group, whereas CK-B by immunoinhibition showed
identical variation for both the UAP and the non-IHD/SAP
groups. Those authors argued that the reason for this
divergence was that the noise in the CK-B analysis was too
high and the analytical sensitivity too low. This is con-
formed by our investigation, in which the variations of
CK-MB immunoassay measurements were explicitly
small in the non-IHD and the SAP groups. In comparison,
the UAP group divided into two separate groups: one with
variations identical to the combined non-IHD/SAP group,
the other with significantly greater variations. These find-
ings indicate that the new CK-MB assay is able to reflect a
wider spectrum of myocardial tissue damage than are
conventional biochemical markers.

Whether the assay will work in a true clinical situation
with nonselected patients remains to be shown. The first
results from a clinical study suggest that this is so (16).

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