Immunochromemical Determination of CK-MB Isoenzyme in Human Serum: A Radiometric Approach
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The presence of creatine kinase isoenzyme MB (CK-MB) in human serum is an indicator of myocardial injury. In this assay for CK-MB, reagent containing both rabbit antibodies to CK-MM and 125I-labeled sheep antibodies to CK-BB is added to the patient's serum and incubated for 1 h at ambient temperature. Goat anti-rabbit immunoglobulin, conjugated to a mixture of amyllose and polyvinylidene fluoride floccules, is then added. After a 15-min incubation, the mixture is centrifuged, isolating the two M-subunit-containing isoenzymes as insoluble complexes. Because the B-subunit portion of MB is labeled with 125I, the radioactivity in the pellet will be proportional to the amount of MB present. The discarded supernate contains excess sheep 125I-labeled BB antibodies, free or bound to BB isoenzyme if it is present. The preparation and characterization of the isoenzymes and antibodies is explained. Concentrations of CK-MB in sera of patients with and without an acute myocardial infarction were assayed in serial specimens obtained in 103 consecutive admissions to a coronary care unit. The performance of this radiometric procedure compared well with CK-MB electrophoresis, giving a sensitivity of 100% and a specificity of 92%.

Additional Keyphrases: heart disease - myocardial infarction - cutoff value

Creatine kinase (CK; ATP: creatine N-phosphotransferase, EC 2.7.3.2) exists as three isoenzymes, which are dimers composed of two types of monomer subunits, designated M (for skeletal muscle-derived) and B (for brain-derived). The isoenzymes consist of the three possible combinations of the two monomers as represented by the symbols MM, MB, and BB (1). Creatine kinase MB isoenzyme (CK-MB) is found in appreciable amounts only in myocardial tissue (2).

The measurement of CK-MB has been widely accepted in the last few years as one of the most sensitive indicators of myocardial injury (3–6). The current techniques for CK isoenzyme assay have been well reviewed (7–9). The published procedures suffer from one or more of the following problems: poor specificity, low sensitivity, poor reproducibility in the lower ranges of MB, and lack of simplicity.

The present report describes a simple radiometric immunnoassay that is both specific and sensitive for measuring CK-MB in human serum and can be used in the routine diagnosis of acute myocardial infarction.

Materials and Methods
Specimen Procurement and Handling

Sera were routinely obtained from patients on admission to the coronary care unit and subsequently each morning for at least two days. Total creatine kinase and total lactate dehydrogenase activity (LD; EC 1.1.1.27) and their isoenzymes were determined in each specimen. Samples were stored at 4 °C and tested within 24 h or were stored at −25 °C and tested within 24 h after they were thawed.

Clinical Diagnosis

The diagnosis of myocardial infarction was based on the presence of three or more of the four following independent criteria: typical electrocardiographic changes, clinical history, presence of CK-MB, and presence of a "flipped" LD1/LD2 ratio (3). All of the patients included in this study were admitted to the coronary care unit, to rule in or rule out acute myocardial infarction.

Procedures

Enzyme and isoenzyme assay by electrophoresis. Total CK was assayed by the ultraviolet method of Rosalki, which depends on NAD+ reduction (10), with reagents from Roche Diagnostics, Nutley, NJ 07110. Kinetic measurements were performed at 30 °C with a System 5 Clinical Analyzer (Gilford Instrument Laboratories, Inc., Oberlin, OH 44074). We determined total LD by use of the ultraviolet lactate-to-pyruvate method, using reagents from Fisher Diagnostics, Fair Lawn, NJ 07410, with the Gilford System 5 Analyzer.

We separated CK and LD isoenzymes on agarose films with use of reagents and agarose films manufactured by Corning-Aci, Palo Alto, CA 94306. Bands were detected by placing the electropherogram in a fluorescent light box, and the results were confirmed by fluorescent densitometry. A result was considered positive for LD if the LD-1 peak height was greater than or equal to the LD-2 peak height, as recommended by Galen et al. (3), or the area under the curve showed LD-1 to exceed or equal LD-2 ("flipped LD"). A result was considered positive for CK-MB if we saw and confirmed by densitometry a band comigrating with the CK-MB of a control serum (Caltrol II; Calbiochem-Behring Corp., La Jolla, CA 92037).

Purification of the CK isoenzymes. Human skeletal muscle, human heart, and human brain were used as the tissue source for CK-MM, CK-MB, and CK-BB, respectively. The CK isoenzymes were initially purified by ethanol fractionation followed by batch chromatography on DEAE Sephacel (11). The CK-MM obtained was homogenous on polyacrylamide gel electrophoresis, but CK-MB and CK-BB were seen to contain substantial amounts of impurities. The CK-MB and the CK-BB were further purified to homogeneity by preparative electrophoresis on polyacrylamide gel (Canalco apparatus; Miles Laboratories, Elkhart, IN 46515) according to the manufacturer's directions. Antiserum production. We raised antibodies to CK-MM in rabbits by subcutaneously injecting 0.5 mg of purified CK-MM, emulsified in Freund's complete adjuvant, at weekly intervals on a routine basis. Blood was sampled monthly. We raised antibodies to CK-BB in sheep by subcutaneously injecting 2 mg of purified CK-BB, emulsified in Freund's com-

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plete adjuvant, at weekly intervals. Blood was sampled monthly.

**Preparation of insoluble second antibody.** Antibody to rabbit IgG was raised in goats by injecting subcutaneously 2.5 mg of chromatographically pure rabbit IgG (Miles Laboratories, Elkhart, IN 46514), emulsified in complete Freund’s adjuvant, at weekly intervals on a routine basis. The goat anti-rabbit gamma globulin was conjugated to polyvinylidene fluoride (12), and to amyllose particles as described below.

The ammonium sulfate precipitable gamma globulin was coupled to a fraction of potato starch ("Superlose"; AVEBE Corp., Veendam, The Netherlands). This material contains about 80% amyllose and the granule diameter is predominantly less than 125 μm. Covalent bonding was effected by forming the dialdehydes through oxidation with sodium periodate and the subsequent formation of Schiff’s bases with primary amines of the globulin. The bases were reduced with sodium borohydride. The reaction, reviewed in detail by Guthrie (13), later was applied to solid-phase immune reactions with various other polysaccharide matrices by Wilson et al. (14) and Sanderson et al. (15).

Pellets were of the proper consistency when the final second-antibody reagent mixture included polyvinylidene fluoride floccules (10 g/L) and amyllose particles (90 g/L) in Tris buffer (50 mM, pH 7.5, containing 8.5 g of sodium chloride, 1 g of sodium azide, and 5 g of bovine serum albumin per liter). The binding capacity of the polymer-antibody conjugate was about 150 mg of IgG per liter.

**Affinity chromatography purification of antibodies.** We coupled 5 mg of purified CK-BB to cyanogen bromide-activated Sepharose 4B gel (Pharmacia Fine Chemicals, Piscataway, NJ 08854) according to the manufacturer’s directions. Depending on the titers, 2 to 5 mL of anti-CK-BB serum was added to the CK-BB Sepharose gel. The antibodies were eluted from the gel with 3 mol/L ammonium thiocyanate, dialyzed against sodium phosphate buffer (50 mM, pH 7.5), and stored at −25 °C.

**Iodination of purified anti-CK-BB.** Purified sheep anti-CK-BB was iodinated by a Chloramine T method (16), as follows. Fifty microliters of a 1 g/L purified solution of antibody was placed in a 0.3-mL “Reacti-Vial” (Pierce Chemical Co., Rockford, IL 61105) and 5 μL of Na125I (100 Ci/L; Amersham Searle Corp., Arlington Heights, IL 60005) and 5 μL of Chloramine T (5 g/L solution) were added in sequence to the vial. The contents of the reaction vial were mixed for 60 s and 5 μL of sodium metabisulfite (10 g/L solution) was added. After mixing for 30 s, we added 125 μL of a 50 g/L solution of bovine serum albumin in de-ionized water.

The contents of the vial were chromatographed on Sephadex G-100 equilibrated at ambient temperature with Tris buffer (100 mM, pH 7.5, and containing 8.5 g of sodium chloride and 0.2 g of sodium azide per liter). Fractions constituting the first peak of the chromatograph were pooled and stored at −25 °C.

**Preparation of 125I-labeled antibody reagent.** The iodinated anti-CK-BB was diluted to 200 000 cpm per 100 μL in a solution containing, per liter, 20 g of goat IgG (Fraction II; Pel Freeze Biologicals, Inc., Rogers, AR 72756), 10 g of bovine serum albumin, 10 mmol of ethylenediaminetetraacetic acid, 7.8 mmol of phenylmethlysulfonyl fluoride, and 50 mmol of Tris, pH 7.5, containing 8.5 g of sodium chloride and 1 g of sodium azide per liter. A maximum of 50-60% of the total counts were bound by excess CK-MB. As expected, it was found that a certain amount of antibody activity is lost during the affinity chromatography purification and iodination of the purified gamma globulins.

This solution also contains rabbit anti-CK-MM serum at a concentration sufficient to completely bind both CK-MB and CK-MM within a practical range. The top of the range chosen was 3000 μg of purified CK-MM per liter in the presence of 1000 μg of purified CK-MB per liter. The rabbit anti-CK-MM serum was titrated by adding various volumes of this antiserum to 500 μL of heated human serum containing 3000 μg of CK-MM and 1000 μg of CK-MB per liter and incubating for 1 h at ambient temperature. Insoluble anti-rabbit IgG was added, the mixture was incubated for 15 min at ambient temperature, and the tubes were centrifuged to remove insoluble complexes. The supernates were then analyzed by electrophoresis on agarose gels, to test for the absence of iso-enzymes.

**Preparation of CK-MB standards.** The concentration of purified CK-MB was determined by measuring the protein content by the method of Lowry et al. (17), with crystalline bovine serum albumin as the standard. Standards were prepared by diluting the purified CK-MB in heated normal goat serum containing, per liter, 10 mmol of EDTA, 7.6 mmol of phenylmethlysulfonyl fluoride, and 1 g of sodium azide. The heated normal goat serum was used as the "0" standard.

**CK-MB radiometric assay procedure.** Patient’s serum or MB standards (500 μL) were added to 12 × 75 mm polystyrene tubes in duplicate. 125I-labeled antibody reagent (100 μL) was added to all tubes, gently mixed, and incubated for 1 h at ambient temperature. Thoroughly suspended insoluble anti-rabbit IgG (200 μL) was added to all tubes, gently mixed and allowed to incubate at ambient temperature for 15 min. Two milliliters of isotonic saline was added to all tubes, followed by centrifugation at 1800 × g for 10 min to remove insoluble complexes. The supernates were decanted and the pellets resuspended in 2 mL of isotonic saline, vortex-mixed, and re-centrifuged. The supernates were again decanted and the radioactivity of the pellets was counted in a gamma scintillation counter. The concentration of CK-MB in patients’ serum was determined by reading results from the appropriate standard curve. The time required to run the standards and 15 samples in duplicate by the above procedure is about 130 min. The laboratory technologist needs 35 min of “hands on” time.

For analysis of the clinical samples, any serum sample with

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**Fig. 1.** Electropherogram of supernates obtained after treatment of purified CK-MM and CK-MB with various amounts of rabbit anti-CK-MM serum (diluted 70-fold in 20 g/L goat gamma globulin solution) and insoluble goat anti-rabbit gamma globulin

a, CK-MM and CK-MB untreated; b, CK-MM and CK-MB after treatment with 10 μL of diluted antiserum; c, after treatment with 25 μL of diluted antiserum; d, after treatment with 50 μL of diluted antiserum; e, after treatment with 100 μL of diluted antiserum.
total CK activity >600 U/L (Roche CK reagent, 30 °C) was diluted in heated normal goat serum containing 10 mmol of EDTA, 7.6 mmol of phenylmethylsulfonyl fluoride, and 1 g of sodium azide per liter. Twenty serum samples out of a total of 362 had total CK activity greater than 600 U/L.

Results

Purity of Isoenzymes

The purity of the CK-MM, CK-MB, and CK-BB isoenzyme preparations used for antibody production were routinely evaluated by electrophoresis on 70 g/L polyacrylamide gels. Greater than 90% purity was achieved in all cases.

Titration of Rabbit Anti CK-MM Serum

Figure 1 shows an electropherogram of supernates obtained after treatment of purified CK-MM and CK-MB in heated human serum with various volumes of rabbit anti-CK-MM serum as explained in Materials and Methods. When sufficient antiserum was present, the CK-MM and CK-MB isoenzymes were completely removed from the serum.

Standard Curve and Precision

A typical standard curve for the CK-MB radiometric assay is shown in Figure 2. Precision of the assay was evaluated by analyzing a positive control system (Caltrol II) eight times on a given day for intra-assay precision and daily for eight days for inter-assay precision. The CVs for intra- and inter-assay precision were 2.0 and 6.8%, respectively.

Effect of CK-MM and CK-BB on Assay

The results obtained in the radiometric assay for various concentrations of the purified CK-MM and CK-BB isoenzymes are presented in Table 1. Electrophoretic analysis of these mixtures is shown in Figure 3.

We did not detect CK-MM or CK-BB in greater amounts than are found in most pathological sera in the CK-MB radiometric assay. The presence of CK-BB simultaneously with CK-MB in serum, however, can lead to an underestimation of CK-MB concentration due to competition of CK-BB for the limited amount of labeled BB antibodies (see Table 1). Theoretically, then, false-negative results are possible for CK-MB in patients' sera containing substantial amounts of CK-BB.

Linearity of the Assay

Excellent linearity was obtained in the radiometric assay for a positive CK-MB serum diluted serially in heated human serum. Analytical recovery of CK-MB at the different dilutions ranged from 88–100%. The dilution containing 50 µg/mL of CK-MB showed the presence of a trace of CK-MB by electrophoresis, while in the dilutions containing 25 and 12 µg/mL no CK-MB was detected by electrophoresis.

Analysis of Patients’ Data

CK-MB was above normal at admission in a number of patients, as shown in Figure 4. However, selectivity appears to be maximum on post-admission days 1 and 2. In a few cases increased CK-MB was observed on day 3.

The mean and standard deviation (SD) of the serum CK-MB concentrations of all non-myocardial-infarct patients for every day when specimens were collected, and the upper limit of normal was defined as the mean +3 SD. As can be seen in Figure 4, this upper limit of normal was useful in differentiating non-myocardial-infarct from myocardial-infarct patients. The mean concentration and the standard deviation were 3 and 8 µg/L, respectively. Therefore, the normal range (mean +3 SD) for the CK-MB assay was 0–27 µg/L. Further studies would establish a more accurate cutoff point.

Table 1. Influence of CK-MM and CK-BB on the Radiometric Determination of CK-MB

<table>
<thead>
<tr>
<th></th>
<th>MM µg/L</th>
<th>MB µg/L</th>
<th>BB µg/L</th>
<th>Found µg/L</th>
<th>% decrease in apparent MB concn</th>
</tr>
</thead>
<tbody>
<tr>
<td>a.</td>
<td>8000</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>b.</td>
<td>0</td>
<td>0</td>
<td>1000</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>c.</td>
<td>2000</td>
<td>290</td>
<td>0</td>
<td>286</td>
<td>0</td>
</tr>
<tr>
<td>d.</td>
<td>2000</td>
<td>290</td>
<td>25</td>
<td>229</td>
<td>20</td>
</tr>
<tr>
<td>e.</td>
<td>2000</td>
<td>290</td>
<td>50</td>
<td>180</td>
<td>37</td>
</tr>
<tr>
<td>f.</td>
<td>2000</td>
<td>290</td>
<td>200</td>
<td>88</td>
<td>69</td>
</tr>
<tr>
<td>g.</td>
<td>0</td>
<td>290</td>
<td>0</td>
<td>288</td>
<td>0</td>
</tr>
</tbody>
</table>

* See Figure 3 for electrophoretic analysis of these mixtures.
Table 2. Comparison of CK-MB Radiometric Assay and CK-MB and LD Electrophoresis

<table>
<thead>
<tr>
<th>CK-MB ( ^a )</th>
<th>CK-MB ( ^b )</th>
<th>LD1/LD2 ratio pos.</th>
<th>CK-MB ( ^c )</th>
<th>CK-MB ( ^d )</th>
<th>LD1/LD2 ratio neg.</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. patients</td>
<td>52</td>
<td>52</td>
<td>45</td>
<td>47 ( ^e )</td>
<td>47 ( ^e )</td>
</tr>
<tr>
<td>Sensitivity</td>
<td>100%</td>
<td>100%</td>
<td>87%</td>
<td>92%</td>
<td>92%</td>
</tr>
</tbody>
</table>

\( ^a \) One positive value of CK-MB on any serum specimen was required for the patient to be classified as positive by the radiometric assay. \( ^b \) One non-infarct patient was CK-MB positive by both the electrophoretic and radiometric assays; the diagnosis was acute ventricular failure and renal failure. Three other non-infarct patients were positive by the radiometric assay and negative by electrophoresis; the diagnosis in one case was myocardial ischemia with atrial fibrillation. \( ^c \) Three non-infarct patients had a positive CK-MB band by electrophoresis but were negative by the radiometric assay.

Table 2 summarizes the results obtained when we compared the radiometric CK-MB assay to CK-MB and LD electrophoresis. All myocardial-infarct patients were correctly classified by both CK-MB procedures, while 47 of 51 non-myocardial-infarct patients were correctly classified by either procedure. In the case of LD electrophoresis, in 45 of 52 myocardial-infarct patients and in 3 of 51 non-myocardial-infarct patients a flip was observed. Therefore, the observed sensitivity of CK-MB was 100% by either the radiometric or electrophoretic procedures while the sensitivity of LD electrophoresis was only 87%. However, the specificity of the CK-MB radiometric assay or CK-MB electrophoretic analysis was comparable to that of LD electrophoresis.

Thirty-two sera taken at admission from the 51 myocardial-infarct patients were positive by both CK-MB procedures. In three additional sera, CK-MB was detected by electrophoresis, and in three others by the radiometric assay on the admission specimen. Therefore, the radiometric assay did not identify myocardial-infarct patients earlier than electrophoresis.

Serum from one non-myocardial-infarct patient was MB-positive by both the radiometric and electrophoretic procedures (80 and 474 \( \mu \)g/L on days 0 and 1, respectively). Serum from three other non-myocardial-infarct patients showed the presence of a trace of MB electrophoretically and values within the normal range (23, 21, and 10 \( \mu \)g/L) by the radiometric assay. Serum from three additional non-myocardial-infarct patients showed increased MB (66, 36, and 39 \( \mu \)g/L) in the radiometric assay while none was detected by electrophoresis (see footnotes to Table 2). In none of these patients' samples was BB present.

**Discussion**

The reaction of CK isoenzymes with antisera prepared by injecting purified CK-MM or CK-BB in sheep, goats, and rabbits has been thoroughly studied (18–26). Antibodies produced against CK-MM do not cross react with CK-BB, and vice versa. Nevertheless, as would be expected from its hybrid structure, CK-MB reacts with antibodies to either CK-MM or CK-BB.

For the present work we used antibodies to CK-MM to isolate the MM and MB isoenzymes on the surface of an insoluble second antibody, thus separating them from any BB isoenzyme in the patient's serum. In the presence of excess anti-MM-serum, the precipitation of all the MM and MB isoenzymes from serum can be assured, as we confirmed by electrophoretic analysis of the supernates. Radiolabeled antibodies to CK-BB were used as the detecting system for the isolated CK-MB, thus providing a highly specific procedure, because only the hybrid isoenzyme can simultaneously react with both MM and BB antibodies. In those unusual cases of myocardial-infarct patients' sera containing both CK-MB and CK-BB, the two isoenzymes will compete for a limited amount of labeled antibodies, as demonstrated in Table 1. Consequently, the observed value for the concentration of CK-MB will be lower than the actual value. In very rare myocardial-infarct patients whose sera contains more CK-BB than CK-MB, the values of CK-MB given by the radiometric assay could be below the cutoff value. Therefore, in cases where serial samples from patients suspected of having had a myocardial infarction show an elevated or "flipped" LD pattern with a negative CK-MB by the radiometric assay, electrophoresis could be used to investigate the simultaneous presence of CK-MB and CK-BB.

Two immunochemical techniques for determining CK-MB,
immunoinhibition of the M-subunit and radioimmunoassay, have been extensively studied (18–25). In the immunoinhibition method, an antibody to the MM isoenzyme inhibits M-subunit activity. This methodology suffers from several problems: (a) difficulty in obtaining complete inhibition of the M-subunit activity, (b) severe interference by the BB isoenzyme because the assay is more sensitive to BB than MB, (c) interference from adenylate kinase (EC 2.7.4.3), and (d) low sensitivity for MB (18–20). The first three factors limit the specificity of these assays.

CK-BB isoenzyme strongly reacts in the radioimmunoassays for the determination of CK-MB reported in the literature. The published procedures are specifically designed to measure the presence of the B subunit (21–25). However, the reality of the presence of CK-BB in a variety of pathological conditions has been well documented (25, 27–32). Homburger and Jacob (25) showed that results by a B-subunit radioimmunoassay were less specific than electrophoresis for the diagnosis of myocardial infarction. In his study, sera from patients with a various non-cardiac medical and surgical diagnoses had increased concentration of B-subunit. This can limit a B-subunit radioimmunoassay in the context of diagnosing myocardial infarct in the patient population admitted to a coronary unit. However, because of the specificity for MB in this radiometric assay, the presence of CK-BB will not cause a false positive in this patient population (see Table 1). Several authors have reported earlier identification of myocardial infarction and (or) higher clinical sensitivity using a B-subunit radioimmunoassay when compared with electrophoresis. In the present study and that of Kwong et al. (33), an earlier diagnosis than with electrophoresis could not be made with use of this radiometric assay for CK-MB. The sensitivity in these two studies was 100% by both methodologies. However, it is important to stress that the reproducibility of this test for low concentrations of CK-MB is excellent, as demonstrated by a CV of 3.1% for intra-assay precision and 10.5% for inter-assay precision at a concentration of 23 µg/L, which is close to the decision level. This could be helpful in providing the cardiologist with a more reliable diagnosis, as shown by Kwong et al. (33).

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References