Immunochemical Extraction and Automated Measurement of Plasma Creatine Kinase MB Isoenzyme and Creatine Kinase MB₂ Isoform

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Measurement of creatine kinase MB (CK-MB) and its isoforms CK-MB₂ and CK-MB₁ are now applied in the diagnosis of acute myocardial infarction (AMI). The most common approach for analysis includes RIA, IRMA, and electrophoresis, all of which may be time-consuming. This study examines determination of CK-MB and CK-MB₂ by a rapid immunochemical extraction method followed by an automated measurement for both analytes. The automated method was sensitive to 2 U/L, linear to 180 U/L, and gave excellent interassay precision (<10% CV). Interference studies indicated that bilirubin, hemolysis, and lipemia caused analytical problems as did the presence of high activities of other CK isoenzymes, notably CK-MM and CK-BB, requiring dilution of samples prior to analysis. Application of immunochemical extraction gave a reference in-

terval of CK-MB (0–2.5 U/L) and CK-MB₂ (0.1–1.4 U/L) for blood donors (20–60 years), peak levels for ruled-out AMI patients of CK-MB (0.5–7.3 U/L) and CK-MB₂ (0.3–4.9), peak levels for ruled-in AMI patients of CK-MB (80–174 U/L) and CK-MB₂ (80–155 U/L). Coronary artery bypass patients (n = 24) and all trauma patients (n = 14) also demonstrated elevations in CK-MB and CK-MB₂, whereas only five of the trauma patients demonstrated increased CK-MB by IRMA. In patients (n = 7) having increased total CK and normal CK-MB by IRMA, the extraction assay for CK-MB and CK-MB₂ yielded increased values in all patients. This new approach to CK-MB and CK-MB₂ analysis can be performed within 30 minutes of sample receipt. *J. Clin. Lab. Anal.* 11:163–168, 1997. © 1997 Wiley-Liss, Inc.

Key words: enzyme analysis; cardiac arrest; automation; interference study

INTRODUCTION

Over 4 million patients are admitted to the hospital each year in the United States for suspected acute myocardial infarction (AMI). Of these, 13 million are diagnosed with AMI, resulting in ~600,000 deaths per year (1).

The diagnostic criteria as established by the World Health Organization (WHO) for the diagnosis of AMI include a history of chest pain, evolutionary changes on the electrocardiogram (ECG), and elevated cardiac enzyme levels. Among the cardiac enzymes, creatine kinase MB (CK-MB) has become the most important serum enzyme test in the diagnosis of AMI (2). The diagnostic sensitivity of serial CK-MB measurements is 98% or better and the diagnostic specificity is 97% or greater.

However, CK-MB measurements are not effective early markers for AMI, as serum levels often take 6–8 hours to reach abnormal levels. Another shortcoming of CK-MB is that levels return to normal within 24–36 hours of the infarct, and indeed levels would be normal should patient admission be delayed for 2 or more days post-AMI.

Recently, the analysis of CK-MM and CK-MB isoforms

have shown that they are more sensitive during the initial 2–6 hours after onset of AMI than CK-MB. There are three CK-MM and two CK-MB isoforms that are present in the serum (3). Each originate from a single isoform found in tissues and when skeletal muscle or myocardial cells become necrotic from disease or normal metabolic events, the tissue isoforms CK-MM₃ and CK-MB₂ are released into the circulation. The action of serum carboxypeptidase N converts CK-MM₃ to CK-MM₂ and then to CK-MM₁ and CK-MB₂ to CK-MB₁ by cleavage of the C-terminal amino acid lysine of the M-subunit (4).

Although measurement of MM isoforms provides early diagnosis, the clinical specificity is low because neither the CK-MM isoenzyme nor its isoforms are specific to the myocardium (5). In contrast, the myocardium contains a higher concentration of CK-MB₂ isoform than skeletal muscle. Consequently, increases in the ratio of CK-MB₂/

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CK-MB₁ with an overall increase in CK-MB/total CK (sometimes referred to as a Relative Index) are highly specific markers for early AMI (6). A high rate of CK-MB₂/CK-MB₁ with a normal Relative Index is more indicative of acute skeletal muscle injury.

Various techniques used to measure CK-MB and CK-MB₂ have involved the use of radioimmunoassay, immunoradiometric (IRMA) assays, and electrophoresis, all of which have been time-consuming and have not been satisfactory in the timely diagnosis of AMI. Further, these assays have been inadequate in providing rapid data concerning enzyme clearance when thrombolytic therapy has been used during early AMI. This study centered on the immunochemical extraction and rapid automated measurements of CK-MB isoenzyme and CK-MB₂ isoform and their application in the diagnosis of AMI, ruled-out AMI, coronary artery bypass graft recipients, trauma with and without cardiac contusion, and events where patients had elevated total CK measurements with normal values of CK-MB.

MATERIALS AND METHODS

Clinical Specimens

All samples were collected by venipuncture and EDTA was employed as an anticoagulant. Samples were usually analyzed within 2 hours; however, when the assay was delayed, samples were stored at 4°C. In some situations when samples were stored frozen for > 7 days, 20 mmol/L of n-acetyl cysteine were added as a CK activator.

Blood samples were collected from the following categories of patients: (1) blood donors, age range 20–60 years (n = 50), (2) patients in whom AMI was *ruled-out* based on clinical impression and available biochemical data (n = 47), (3) patients in whom AMI was *ruled-in* based on clinical impression and available biochemical data (n = 40), (4) coronary artery bypass graft recipients (n = 24), (5) trauma patients with and without cardiac contusion (n = 14), (6) patients who had elevated total CK with normal CK-MB concentrations as measured by an immunoradiometric assay (n = 7). In all patient groups, serial blood samples were obtained over the duration of hospitalization.

Measurements

Total CK activity in U/L was measured using the Hitachi 736 analyzer where the production of NADPH was followed photometrically at 376 and 340 nm.

CK-MB EU/L was measured routinely using an IRMA supplied by International Immunoassay Laboratories (Santa Clara, CA). The assay took ~3 hours to complete, the units were EU/L (equivalent units/L, where 1 EU/L = 2.4 ng/mL CK-MB), and values < 3.7 EU/L were considered normal.

CK-MB and CK-MB₂ activities in U/L were measured using reagents from Roche Diagnostic Systems (Nutley, NJ) on

a Cobas Fara II analyzer after immunochemical extraction using reagents (IMPRES-MB-X) supplied by International Immunoassay Laboratories. When using the Cobas Fara II analyzer, a sample volume of 40 µl was used and a factor of 1256 was applied to calculations that included a dilution factor of 1:25. These factors were part of a modification of the CK assay on the analyzer to achieve a high degree of analytical sensitivity with acceptable precision.

Briefly, CK-MM was extracted from patient plasma samples (100 µL) with reagent A (25 µL), which contained magnetizable particles onto which anti-CK-MM antibody was immobilized. This reagent was optimized for removal of CK-MM with a minimum removal of CK-MB and hence left CK-MB and CK-BB in the sample. Another aliquot (100 µL) of the patient sample was treated with reagent B (25 µL), which had been optimized to remove M subunit isoenzymes of CK. Reagent B contained magnetizable particles coated with an antibody to remove M subunits and left CK-BB, atypical CKs, and adenylate kinase in the treated sample.

Both tubes containing reagents A and B were placed on a magnetic rack after a 15-minute incubation period during which they were gently rotated to keep the particles in suspension. Clear supernatant was removed for the measurement of residual enzyme activity and the difference in activities between tubes A and B gave a measurement of CK-MB (U/L).

A third aliquot (100 µL) of patient sample was treated with reagent C (25 µL), which contained the same contents as reagent A, and also a monoclonal antibody, which reacts with serum-specific M subunit of CK-MB. Tube C was similarly gently rotated for 15 minutes and then placed on a magnetic rack. The enzymatic activity of the reagent C tube reflected the contribution of CK-MB₂ isoform, CK-BB, atypical CKs, and adenylate kinase. Thus the difference in enzyme activity between tubes C and B gave that due to the isoform CK-MB₂ (U/L).

Precision Studies

Total CK, CK-MB, and CK-MB₂ by immunochemical extraction were monitored for precision using Technicon Test Point Control I diluted 1:2, 1:20, 1:30 and 1:50 using enzyme diluent supplied by Clinical Chemistry Consultants (Temple City, CA). CK-MB precision by IRMA was followed using controls supplied by Baxter Diagnostics (Deerfield, IL).

Interference Studies

All interferents were added to a pooled serum collected from patients in whom the diagnosis of myocardial infarction had been confirmed both by clinical confirmation and biochemical means using the CK-MB IRMA assay. The working pool had a CK-MB value of 13.7 EU/L (reference interval 0–3.7 EU/L).

RESULTS

Using the modified CK assay on the Cobas Fara II, it was possible to obtain an excellent method that was linear to 180 U/L and had a sensitivity of 2 U/L. Further, both intra- and inter-assay precision values were acceptable when studying low to high concentrations of the isoenzyme (Table 1).

In terms of interference for the CK-MB immunoextraction assay, bilirubin, hemolysis, and lipemia demonstrated considerable negative interference at levels of 11.83 mg/dL, 250 mg/dL, and a factor of +2, respectively. CK-MM activity did not appear to be a problem until values of 1,000 U/L were reached. CK-BB values of 400 U/L or greater could interfere significantly giving a positive bias (Table 2).

Interference in the CK-MB₂ immunoextraction assay demonstrated negative interference with bilirubin, hemolysis, and lipemia at 11.83 mg/dL, 500 mg/dL and a factor +2, respectively. Similarly to the CK-MB assay, CK-MB₂ isoform testing demonstrated positive interference at CK-MM levels at 1,000 U/L or greater. In terms of CK-BB, positive interference of +12% or greater were demonstrated at CK-BB activity levels of 40 U/L or greater (Table 3).

Table 4 gives the range values (lowest to highest) for total CK, CK-MB (EU/L) and CK-MB (U/L), CK-MB₂ (U/L) by immunoextraction. The values reported for the blood donors set a baseline and with AMI ruled-out patients (admit values) both CK-MB (U/L) and CK-MB₂ (U/L) appeared to be slightly elevated. Peak values for AMI ruled-out patients demonstrated that some patients shared elevations above the reference interval, i.e., CK-MB 7.3 U/L, CK-MB₂ 4.9 U/L, although it should be mentioned that the peak values of total CK were also elevated up to 319 U/L.

In terms of the admit values for AMI ruled-in patients, there was a wide range for total CK (24-2328 U/L), CK-MB (EU/L) and both CK-MB (U/L) and CK-MB₂ (U/L). However, peak values of the analytes were clearly elevated above the general reference interval in all patients.

TABLE 1. Precision of the CK-MB Assay Performed Using the Cobas Fara II

Intraassay precision (n = 10)	
CK-MB (U/L)	2CV (%)
3.2	12.4
6.2	3.7
9.5	1.6
90.0	1.9
175.0	1.7
Interassay precision (n = 24)	
CK-MB (U/L)	2CV (%)
3.2	27.4
6.2	17.9
9.5	17.3
90.0	7.2
175.0	7.2

TABLE 2. Interference in the CK-MB Immunoextraction Assay

CK-MB Interferent	Concentration	% Change in CK-MB value
Bilirubin	(11.83 mg/dL)	-38.8
	(50 mg/dL)	-0.3
	(250 mg/dL)	-26.9
	(500 mg/dL)	-30.5
Lipemia	(+2 classification)	-61.3
CK-MM	(250 U/L)	+5.0
	(500 U/L)	+6.0
	(1000 U/L)	+14.0
	(2000 U/L)	+45.7
CK-BB	(4000 U/L)	+120.0
	(40 U/L)	-0.9
	(80 U/L)	-4.8
	(200 U/L)	+3.1
	(400 U/L)	+13.0
	(800 U/L)	+29.0

For coronary artery bypass patients, the immunochemical extraction assays (CK-MB and CK-MB₂) clearly showed differences from the reference interval as did trauma patients and those patients who had normal CK-MB (EU/L) with increased total CK (Table 4).

Table 5 gives peak isoenzyme values for the various analytes on 14 trauma patients along with the associated diagnosis. Clearly, all CK-MB (U/L) and CK-MB₂ (U/L) are elevated whereas the CK-MB (EU/L) assay gave 5 of the 14 with increased isoenzyme.

In Table 6, it can be seen that the group of patients who had increased total CK and normal CK-MB (EU/L) all demonstrated increased values of CK-MB (U/L) and CK-MB₂ (U/L) for the peak sample obtained.

DISCUSSION

Certainly cardiac arrest is a leading cause of death placing an enormous burden on hospital emergency rooms. However,

TABLE 3. Interference in the CK-MB₂ Immunoextraction Assay

CK-MB ₂ Interferent	Concentration	% Change in CK-MB ₂ value
Bilirubin	(11.83 mg/dL)	-4.8
	(50 mg/dL)	-0.8
	(250 mg/dL)	-5.0
	(500 mg/dL)	-10.0
Lipemia	(+2 classification)	-43.6
CK-MM	(250 U/L)	+4.6
	(500 U/L)	+6.0
	(1000 U/L)	+18.5
	(2000 U/L)	+46.0
CK-BB	(4000 U/L)	+53.0
	(40 U/L)	+12.6
	(80 U/L)	+17.9
	(200 U/L)	+17.5
	(400 U/L)	+13.2
	(800 U/L)	+22.2

TABLE 4. Range Interval-Values for Total CK, CK-MB (EU/L), CK-MB (U/L), and CK-MB₂ (U/L)

	Total CK (U/L)	CK-MB (EU/L)	CK-MB (U/L)	CK-MB ₂ (U/L)
Blood donors (n = 50)	30–180	0–3.7	0–2.5	0.1–1.4
Ruled-out AMI (n = 47) (admit values)	21–202	2–3.5	0.1–3.7	0.6–3.3
Ruled-out AMI (n = 47) (peak values)	24–319	2–2.4	0.5–7.3	0.3–4.9
Ruled-in AMI (n = 40) (admit values)	24–2,328	2.2–57	0.7–75.3	0.4–51.3
Ruled-in AMI (n = 40) (peak values)	135–4,210	3.9–119	80–174	80.0–155
CABG patients (n = 24)	83–4,700	1.5–148	3–218	4–189
Trauma patients (n = 14)	192–10,540	0.5–23.5	3–219	1–85
Increased total CK (n = 7) (normal CK-MB EU/L)	82–3,218	0.2–3.6	1.3–73	0.1–45.7

< 30% of the patients admitted to a coronary care unit are subsequently found to have AMI (7, 8). The cost of caring for patients in whom myocardial infarction has been ruled out has been estimated to be nearly \$4 billion dollars per year and hence there have been suggestions that they should be cared for in a less intense environment (9).

Given that clinical history and electrocardiogram can be nonspecific and that current CK-MB assays may not offer the laboratory confirmation of AMI, there has been recent interest in the measurement of CK-MB isoforms and their application in early diagnosis. Indeed, it has been reported that a CK-MB₂ isoform level of > 1 U/L with a CK-MB₂/CK-

MB₁ ratio of > 1.5 provides improved sensitivity and specificity for the diagnosis of AMI within 6 hours after the onset of symptoms when compared to current assays for CK-MB isoenzyme (10).

In order to measure CK-MB isoforms and report data to the emergency room in a timely manner, most laboratories have had to employ a high voltage electrophoretic technique. There are, of course, problems with such an approach, e.g., maintenance, gel quality, and the need to purchase equipment. Hence the purpose of this study was to examine the possibility of applying an immunochemical extraction technique for the isolation of CK-MB isoenzyme and CK-MB₂ isoform from

TABLE 5. Clinical Details for Trauma Patients (Peak Enzyme Values)

Patient number	Peak total CK (U/L)	Peak CK-MB (EU/L)	Peak CK-MB (U/L)	Peak CK-MB ₂ (U/L)	Diagnosis
1	4,175	3.5	51.2	16.8	Motor vehicle accident Cardiac contusion ruled out
2	1,289	3.7	18.0	12.0	Motor vehicle accident Coma/M.I. ruled out
3	853	12.6	32.5	27.0	Motor vehicle accident Cardiac contusion
4	1,212	3.7	9.8	7.1	Motor vehicle accident Skull fracture
5	9,048	24.1	23.0	14.0	Sepsis/chest pain/ Surgery/diabetes
6	1,299	3.7	40.5	34.8	Motor vehicle accident/lung puncture/broken ribs
7	548	2.7	7.2	2.2	Broken hip/Alzheimer's disease
8	2,931	6.6	56.4	51.0	Hepatitis B/liver transplant
9	10,870	4.7	107.1	32.0	Rhabdomyolysis/drug abuse cocaine overload
10	2,875	9.1	224.0	46.2	Cocaine-induced cardiac ischemia M.I. ruled out
11	2,238	2.6	22.0	11.0	EKG normal/stab wounds
12	689	3.4	10.0	8.8	Multiple injuries/15 foot fall from rocks
13	808	2.6	8.0	2.2	Motor vehicle accident Alcohol 200 mg/dL
14	4,130	3.2	54.9	35.1	Cranial trauma/pneumonia

TABLE 6. Clinical Details for Patients With Increased Total CK and Normal CK-MB (EU/L) as Measured by Immunoradiometric Assay

Patient number	Peak total CK (U/L)	Peak CK-MB (EU/L)	Peak CK-MB (U/L)	Peak CK-MB ₂ (U/L)	Diagnosis
1	2,185	2.6	39.2	20.0	Multiple sclerosis/septicemia
2	3,300	3.4	74.9	46.9	Rhabdomyolysis
3	982	2.7	19.0	12.6	Alcoholism
4	2,596	2.4	21.5	12.0	Parkinson's disease
5	658	3.1	9.8	7.4	hyponatremia Coronary artery spasm hip replacement
6	836	2.4	17.2	1.6	Chest pain/prostatic cancer
7	475	3.7	49.8	18.2	Hypertension/renal insufficiency

plasma and finally quantitate each by a rapid automated technique using a biochemical analyzer which is widely available.

Before the study could be initiated, it was essential that a method for enzymatic measurement of low levels of CK-MB and CK-MB₂ be established. This was successfully achieved using reagents supplied for the measurement of total creatine kinase on the Cobas Fara II, which usually has an analytical range of up to 1,200 U/L for the enzyme. Modifications included increased dilution of samples to 1:25, increased read times over the kinetic aspects of the assay, which resulted in a final factor for the system of 1256. As the results indicate, a creatine kinase assay was developed that had low sensitivity and linearity to 180 U/L. Further both intra- and inter-precision studies show that it was reproducible at anticipated normal levels of enzyme activity, which is usually considered to be up to 5 U/L for CK-MB.

In establishing the immunoextraction assays for CK-MB and CK-MB₂, it was found that high bilirubin levels will lead to negative bias as will hemolysis, although the CK-MB₂ assay seemed to be less sensitive to these interferents. The presence of lipemia appeared to affect both analytes equally.

There was significant interference from CK-MM above 1,000 U/L for both CK-MB and CK-MB₂, and hence samples needed to be diluted before testing. This is obviously a drawback to an immunochemical approach as the laboratory would be required to perform a total creatine kinase determination before CK-MB and CK-MB₂ analysis. Interference from CK-BB isoenzyme appeared to be negligible in the CK-MB assay to values > 400 U/L; however, the CK-MB₂ isoform gave a constant interference between 40–400 U/L. These interferences obviously relate to steps in the immunoextraction techniques, especially in the CK-MB assay where immobilized CK-MM antibody is added, and hence elevated levels of total CK (predominantly CK-MM) would lead to antigen excess and an over-loading effect. Apparently the CK-MB₂ assay is even more sensitive to elevations in total CK, as the manufacturer recommends dilution of samples containing levels > 500 U/L. Again this is related to the CK-MB₂ assay containing a monoclonal antibody that reacts with the serum-specific M subunit of CK-MB. Therefore before analysis of any

clinical samples was conducted, the total CK value was carefully considered.

It was established that when employing an immunochemical approach, the reference interval for CK-MB is 0–2.5 U/L and for CK-MB₂ is 0.1–1.4 U/L. For 47 patients ruled-out for AMI, hospital admission values gave slightly higher values up to 3.7 U/L for CK-MB and up to 3.3 U/L for CK-MB₂ with peak values (defined as highest total CK measured) of 7.3 U/L and 4.9 U/L, respectively. This obviously reflects the corresponding increased total CK (see Table 4) and the data is useful in establishing ranges for hospitalized non-cardiac patients.

For patients presenting with AMI, hospital admission values of up to 75 U/L for CK-MB and 51 U/L for CK-MB₂ were observed, and on the same group a peak range of 80–174 U/L and 80–155 U/L was obtained, respectively. These values corresponded well with both the total CK and CK-MB (EU/L) enzyme and protein mass measurements and indicate that both CK-MB and CK-MB₂ by immunoextraction could be obtained in a more rapid time frame than traditional immunoassays for CK-MB, i.e., 45 minutes as opposed to 3 hours.

It was not surprising that elevations in CK-MB and CK-MB₂ by immunoextraction were found in coronary bypass graft patient samples, and this was reflected in the same 24 patients who had measurements of total CK and CK-MB (EU/L). Also, trauma patients gave a similar pattern for all analyte ranges. However, when the peak enzyme values were tabulated for these trauma patients (Table 5), it was apparent that the total CK was elevated in all cases. Only five had elevated CK-MB (EU/L), whereas abnormal values were obtained in all patients when using the CK-MB and CK-MB₂ immunoextraction method. Clearly in these clinical situations, CK-MB (EU/L) is the best indicator of true cardiac involvement when the diagnosis is considered (Table 5).

A common question asked of the clinical laboratory is whether or not one can comment on the aspect of elevations of total creatine kinase when mass measurement of CK-MB are normal. In this study, a selection of seven patients was made covering this type of situation and the samples were assayed for CK-MB and CK-MB₂ without knowledge of the clinical diagnosis. In all patients these analytes were elevated

well above the reference intervals established in this study. Reference to the clinical diagnosis had ruled out cardiac involvement (Table 6) and points to a problem with the immunoextraction approach.

In conclusion, this study examined the application of an immunochemical extraction and automated measurement for plasma CK-MB isoenzyme and CK-MB₂ isoform. The assay is useful in the rapid determination of these analytes post-AMI and in other clinical situations. It tends to suffer from interference from other CK isoenzymes, particularly CK-MM, despite pre-sample dilution in cases where the total CK is elevated. However, this is an emerging field that offers much hope for the future because application of rapid CK-MB isoenzymes and isoform analysis will enable patients to be excluded from unnecessary expensive medical care.

REFERENCES

1. Foreback C: Biochemical diagnosis of myocardial infarction. *Henry Ford Hosp Med J* 39:159–164, 1991.
2. Lee TH, Goldman L: Serum enzyme assays in the diagnosis of acute myocardial infarction. *Ann Intern Med* 105:221–233, 1986.
3. Wu AHB: Creatine kinase isoforms in ischemic heart disease. *Clin Chem* 35:7–13, 1989.
4. George S, Ishikawa Y, Perryman MB, et al.: Purification and characterization of naturally occurring and in vitro induced multiple forms of creatine kinase. *J Biol Chem* 259:2667–2674, 1984.
5. Wu AHB, Gornet TG, Wu VH, et al.: Early diagnosis of acute myocardial infarction by rapid analysis of creatine kinase isoenzyme-3 (CK-MM) subtypes. *Clin Chem* 33:358–362, 1987.
6. Harber CC, Wu AHB: Early diagnosis of acute myocardial infarction (AMI) upon initial hospital admission using CK-MB₂ isoform analysis. *Clin Chem* 36:1128, 1990 (Abstract).
7. Weingarten SR, Ermann B, Riedinger MS, Shah PK, Ellrodt HG: Selecting the best triage rule for patients hospitalized with chest-pain. *Am J Med* 87:494–500, 1989.
8. Fesmire FM, Peroy RF, Wears RL, Malmath TL: Risk Stratification according to the initial electrocardiogram in patients with suspected acute myocardial infarction. *Arch Intern Med* 149:1294–1297, 1989.
9. Seker HP: Coronary care unit triage decision aids: How do we know when they work? *Am J Med* 87:491–493, 1989.
10. Puleo PR, Meyer D, Wathen C, et al.: Use of a rapid assay of subforms of creatine kinase MB to diagnose or rule out acute myocardial infarction. *New Engl J Med* 331:561–566, 1994.