LABORATORY INVESTIGATION

REPERFUSION

Early detection of myocardial reperfusion by assay of plasma MM–creatine kinase isoforms in dogs*

STEPHEN R. DEVRIES, M.D., BURTON E. SOBEL, M.D., AND DANA R. ABENDSHEIN, PH.D.

ABSTRACT To determine whether myocardial reperfusion can be detected promptly by changes in profiles of isoforms of MM–creatine kinase (CK) in plasma, coronary occlusion was induced in 30 conscious dogs and reperfusion was initiated after 1, 2, 3, or 4 hr in 21. The myocardial isoform of MM-CK, MM$_{A}$, was quantified in serial plasma samples by chromatofocusing. Before coronary occlusion, MM$_{A}$ comprised 13 ± 7% (SD) of the total CK activity in plasma. The percentage of MM$_{A}$ (MM$_{A}$ %) was elevated before reperfusion, but increased markedly and consistently to a peak of 52 ± 13% ($n = 21$) between 30 min and 1 hr after the time of onset of reperfusion. The rate of increase in MM$_{A}$ % was significantly faster with reperfusion at 1 hr (1.44 ± 0.42% min$^{-1}$), 2 hr (1.28 ± 0.45% min$^{-1}$), or 3 hr (1.02 ± 0.27% min$^{-1}$) (p < .001), but not with reperfusion at 4 hr (0.48 ± 0.34% min$^{-1}$) compared with the rate in nonreperfused control dogs (0.29 ± 0.09% min$^{-1}$). Furthermore, the rate of increase in MM$_{A}$ % was neither influenced by peak total CK activity (r = -.1) nor dependent on infarct size measured histochemically 24 hr after coronary occlusion (r = -.03). The time from coronary occlusion to the peak of MM$_{A}$ % was reduced by reperfusion at 1 to 3 hr compared with control, but this index was not identified as rapidly as the rate of increase in MM$_{A}$ %. Accordingly, characterization of the rate of increase in MM$_{A}$ % in plasma when reperfusion occurs early after the onset of myocardial infarction permits prompt, reliable, and noninvasive detection of myocardial reperfusion.


INTERVENTIONS designed to restore blood flow to ischemic myocardium are undergoing extensive evaluation. Because the potential benefit of reperfusion is highly dependent on its prompt implementation, intravenous administration of thrombolytic agents has been explored as a means of rapidly effecting reperfusion. Delineation of the relative efficacy of specific thrombolytic agents and evaluation of each requires sensitive and reliable methods for noninvasive detection of reperfusion. Conventional noninvasive indexes of reperfusion such as abrupt resolution of chest pain, resolution of electrocardiographic ST segment changes, and accelerated appearance and peaking of total and MB–creatine kinase (CK) activities in plasma are neither sufficiently sensitive nor reliable as criteria of reperfusion. Although coronary angiography is reliable, it is expensive, invasive, and not always immediately available.

We and others have shown that MM-CK released by myocardium undergoing infarction is modified biochemically to yield subforms with different isoelectric points (isoforms).

In dogs subjected to coronary artery occlusion, the predominant isoform in myocardium, MM$_{A}$ (pI = 7.91), is converted quickly and sequentially to two other isoforms, MM$_{B}$ (pI = 7.74) and MM$_{C}$ (pI = 7.51), in plasma. Conversion in concert with disappearance of each isoform from the circulation results in a consistent, time-dependent change in the percentage of total MM-CK contributed by each isoform. Furthermore, because the percentage of MM$_{A}$ in plasma is normally low, isoform profiles are altered markedly by egress of modest amounts of MM$_{A}$ from myocardium, providing a sensitive and very early index of recent myocardial damage. A distinctive alteration of plasma isoform profiles was also shown to be useful as a sensitive, early marker of myocardial infarction in patients.

From the Cardiovascular Division, Washington University School of Medicine, St. Louis.

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Address for correspondence: Stephen R. Devries, M.D., Cardiovascular Division, Washington University School of Medicine, 660 South Euclid Avenue, Box 8086, St. Louis, MO 63110.

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Because consistent changes in the proportions of MM-CK isoforms are manifest in plasma early after myocardial infarction, we postulated that analysis of plasma isoform profiles might permit prompt detection of myocardial reperfusion. Accordingly, the present study was undertaken to characterize changes in isoform profiles in plasma after myocardial reperfusion in dogs and to determine whether changes in isoform profiles early after the onset of reperfusion differ from those after persistent occlusion and can serve to identify successful recanalization.

Methods

Animal preparations. Thirty-nine male mongrel dogs weighing 15 to 28 kg were premedicated with acepromazine maleate (0.5 mg/kg sc) and atropine sulfate (0.04 mg/kg sc). Anesthesia was induced with sodium pentobarbital (30 mg/kg iv) and respiration was maintained with a volume ventilator supplying oxygen-enriched room air. A venous blood sample was obtained before surgery for determination of total CK activity in plasma.

An aseptic left thoracotomy was performed in the fourth intercostal space of each dog and the heart was suspended in a temporary pericardial sling. For myocardial reperfusion studies, a balloon occluder was placed around the left circumflex coronary artery 2 to 3 cm from the origin in 30 dogs. The occluder was fabricated by the method of D. McKowen and D. Franklin (Dalton Research Lab, Columbia, MO). Briefly, the distal end of a No. 8F feeding tube (Bard 3642) was closed to an airtight seal with No. 3 silk suture tied in a square knot. While air pressure was applied to the open end of the tube with a syringe, the closed end was submerged in boiling water until the heated segment dilated to form a narrow balloon. The deflated balloon was wrapped around the vessel with the loop secured by tying the free ends of the No. 3 suture around the tube just beyond the flattened segment. Care was taken to choose a balloon length that did not constrict the vessel. The tubing was sutured to the epicardium at several points to prevent the occluder from distorting the vessel. A 20 MHz pulsed Doppler flow probe (C. Hartley, Baylor College of Medicine, Houston, TX) was placed just proximal to the occluder. In nine dogs that would be subjected to coronary artery occlusion without reperfusion, snare made from 20 pound test nylon line within a polyethylene tube (PE 240) were looped around the vessel. The occluder or snare tubing and flow probe wires were exteriorized through the fifth intercostal space. The chest was closed in layers and evacuated. An external jugular vein was catheterized with silicon rubber tubing filled with heparin. Each dog was given ampicillin (15 mg/kg im) and morphine (0.25 to 1.0 mg/kg im) twice daily for 3 days after surgery.

Experimantal protocol. One or two weeks after surgery, when total CK activity in plasma had declined to preoperative baseline values, 200 ml of venous blood was withdrawn from the jugular catheter and stored at 4°C in a sterile pouch containing 6 mM sodium citrate anticoagulant, 10 mM adenosine, and 5% dextrose (Travenol, CPDA-1). Blood was replaced with an equal volume of 0.9% saline. Twenty-four to forty-eight hours after blood had been collected, each dog was placed in a sling permitting standing or rest with minimal restraint. The electrocardiogram was monitored with limb leads applied with rubber straps. The flow probe on the circumflex coronary artery was activated by a pulsed Doppler flowmeter (Valpey Fisher, Hopkinton, MA, Model VF-1). Morphine (7.5 mg) and lidocaine (30 mg) were given intravenously. Coronary occlusion was induced either by pressurizing the balloon occluder with air until blood flow velocity decreased to zero or by tightening the coronary snare. Coronary occlusion was confirmed by ST segment changes in the electrocardiogram. Additional bolus injections of morphine (2.5 to 5.0 mg iv) were given as needed to preclude discomfort. Reperfusion was implemented 1, 2, 3, or 4 hr after coronary occlusion by deflating the balloon. Reperfusion was verified in each case by the appearance of a flow signal detected with the Doppler probe. Lidocaine (30 mg iv) was administered before reperfusion and subsequently as needed for suppression of ventricular arrhythmias. Within 5 hr after coronary occlusion, each dog was placed in an isolation cage and given free access to water.

Heparinized blood samples (15 U/ml) were obtained before coronary occlusion, before reperfusion, 0.5 and 1 hr after reperfusion, and serially thereafter for a minimum of 13 hr. Blood was drawn to clear the dead space of the venous catheter before acquisition of each sample. Blood samples were replaced with equal volumes of filtered, autologous blood to maintain intravascular volume constant. Samples were centrifuged immediately for 10 min at 1900 g (4°C), and the plasma was supplemented with 2-mercaptoethanol (5 mM final concentration) before storage at −70°C for subsequent assay of isoforms.

Twenty-four hours after coronary occlusion, anesthesia was induced with sodium pentobarbital (30 mg/kg iv). The heart was excised for histochemical determination of infarct size.

Total CK activity and assay of MM-CK isoforms. Total CK activity was measured spectrophotometrically at 37°C with a coupled enzyme system (CK S.V.R. reagents, Calbiochem-Behring, La Jolla, CA) and a Gemeni miniature centrifugal analyzer (Eletro-Nucleonics, Fairfield, NJ). Results are expressed as international units per liter at 30°C.

Isoforms were assayed with a quantitative chromatofocusing technique developed in our laboratory and shown previously to separate isoforms without denaturing CK or producing artifactual sub-bands. A modification of the previously developed procedure permitted direct application to the chromatofocusing column of 1 ml of plasma with total CK activity ranging between 100 and 1000 IU/liter. It involved decreasing the rate of elution of the chromatofocusing column with Polybuffer 94 (Pharmacia, Piscataway, NJ) at pH 6.95 from 1.5 to 0.8 ml/min. When total CK activity in plasma exceeded 1000 IU/liter, a volume of plasma containing 1.0 IU of activity was applied directly to the chromatofocusing column and eluted as described previously. When total CK activity was less than 100 IU/liter, non-CK protein was first removed selectively by affinity chromatography on a Blue Sepharose CL-6B column (Pharmacia) and CK protein was concentrated before chromatofocusing by the modified method. Activities of the three MM-CK isoforms separated by chromatofocusing were expressed as percentages of total CK activity eluted from the column. Absolute activities of isoforms in plasma samples were calculated by multiplying the percentage of each isoform by total CK activity. Total CK activity in plasma obtained from dogs subjected to myocardial infarction consists of greater than 90% MM-CK.

Measurement of infarct size. Hearts were rinsed with saline, the great vessels and atria were removed, and the ventricles were sectioned transversely into five or six slices. Slices were incubated for 10 min at room temperature in a freshly prepared 1% solution of triphenyltetrazolium chloride in 0.2M Tris buffer (pH 7.6) that stains normal myocardium brick red but does not stain infarcted myocardium. Because no evidence of right ventricular infarction was noted in any of the hearts, the right ventricular free wall was removed before weighing of the slices. The outlines of both surfaces and of the unstained areas of
infarct in each slice were traced onto overlying cellophane. A planimeter was used to measure the area of infarct. Infarct weight was calculated as the average fractional area of infarct for both surfaces of a slice multiplied by the weight of the tissue. Infarct size was expressed as the sum of infarct weights from all left ventricular slices.

**Calculation of the rate of increase in plasma MMa expressed as a percentage.** The rate of increase of MMa% (percentage of total CK activity comprising MMa activity) was calculated during the interval showing the greatest change in MMa% and expressed as MMa% min⁻¹. In dogs undergoing reperfusion, the greatest change in MMa% occurred during the first 30 min after reperfusion. In dogs with persistent ischemia, the greatest change in MMa% occurred during a 60 min interval between 0 and 4 hr after coronary occlusion.

**Statistical analysis.** Results are expressed as the mean ± SD. Analysis of variance was used to test the significance of differences in measurements in dogs undergoing reperfusion and nonreperfused control dogs. A p ≤ .05 was considered indicative of a significant difference.

**Results**

Five of the dogs that underwent reperfusion were excluded because they did not sustain any myocardial injury as assessed by the electrocardiogram, plasma CK activity, and morphology of the myocardium at autopsy. Four dogs were excluded because of the occurrence of ventricular fibrillation. Twenty-one dogs comprised the reperfusion group with reperfusion induced 1 hr (n = 3), 2 hr (n = 6), 3 hr (n = 6), or 4 hr (n = 6) after the onset of coronary occlusion. The results from these dogs were compared with data from the nine control dogs with persistent coronary occlusion. Coronary occlusion was verified in control dogs at autopsy.

**CK time-activity profiles.** Total CK activity was 79 ± 49 IU/liter before surgery and 38 ± 12 IU/liter before coronary occlusion. Before reperfusion, total CK activity was not increased compared with baseline values except in dogs undergoing reperfusion at 4 hr. However, total CK activity was consistently elevated 30 min after reperfusion in all dogs. Figure 1 shows the total CK and MMa isomeric profiles in dogs undergoing reperfusion at 2 hr. These profiles are representative of those in dogs that underwent reperfusion at other time intervals. Considerable variation in the contour of the total CK activity curves was evident, reflecting differences in the extent of infarction and probable differences in the apparent rates of enzyme washout. Because total CK activity in canine blood is a measure of the collective activities of MMa, MMb, and MMc, and because MMa comprises greater than 95% of CK in canine myocardium, the activity of MMa in plasma after reperfusion is affected by the amount of CK released from myocardium. Thus, the contours of MMa and total CK time-activity curves were similar (figure 1, A and B). Changes in the activities of MMb and MMc followed sequentially after those in MMa.

**MMa% profiles.** MMa% in plasma was 13 ± 7% before coronary occlusion and 19 ± 10%, 15 ± 8%, 24 ± 6%, and 23 ± 9% before reperfusion at 1, 2, 3, and 4 hr, respectively. Despite the variation in total CK and absolute MMa activity in plasma after reperfusion, profiles of MMa% showed consistent changes (figure 1, C). MMa% was increased markedly 30 min after reperfusion (62 ± 10%, 54 ± 6%, 55 ± 11%, and 36 ± 16% with reperfusion at 1, 2, 3, and 4 hr, respectively). The peak of MMa% was observed at
TABLE 1
CK activity and histochemically measured infarct size after coronary occlusion and reperfusion

<table>
<thead>
<tr>
<th>Dog No.</th>
<th>Duration of ischemia (hr)</th>
<th>Total CK</th>
<th>MM₄₅-CK</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Time to peak (hr)</td>
<td>Peak activity (IU/l)</td>
</tr>
<tr>
<td>1</td>
<td>1</td>
<td>6.0</td>
<td>1158</td>
</tr>
<tr>
<td>2</td>
<td>1</td>
<td>3.0</td>
<td>2195</td>
</tr>
<tr>
<td>3</td>
<td>1</td>
<td>4.0</td>
<td>1453</td>
</tr>
<tr>
<td>Mean ± SD</td>
<td>4.3 ± 1.5</td>
<td>1602 ± 534</td>
<td>1.5 ± 0.0</td>
</tr>
<tr>
<td>4</td>
<td>2</td>
<td>6.0</td>
<td>5648</td>
</tr>
<tr>
<td>5</td>
<td>2</td>
<td>5.0</td>
<td>243</td>
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</tr>
<tr>
<td>9</td>
<td>2</td>
<td>5.0</td>
<td>1411</td>
</tr>
<tr>
<td>Mean ± SD</td>
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<td>1411 ± 2124</td>
<td>2.5 ± 0.0</td>
</tr>
<tr>
<td>10</td>
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<tr>
<td>15</td>
<td>3</td>
<td>5.0</td>
<td>4698</td>
</tr>
<tr>
<td>Mean ± SD</td>
<td>6.3 ± 1.0</td>
<td>2210 ± 1878</td>
<td>3.7 ± 0.3</td>
</tr>
<tr>
<td>16</td>
<td>4</td>
<td>7.0</td>
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<td>21</td>
<td>4</td>
<td>7.0</td>
<td>3796</td>
</tr>
<tr>
<td>Mean ± SD</td>
<td>7.0 ± 0.0</td>
<td>2236 ± 1665</td>
<td>4.8 ± 0.3</td>
</tr>
</tbody>
</table>

*From onset of coronary occlusion.

either 30 min (n = 16) or 1 hr (n = 5) after reperfusion and preceded the time to peak of total CK activity by approximately 2 hr (table 1).

The time course of MM₄₅% in plasma after reperfusion was strikingly different from that in nonreperfused control dogs (figure 2). MM₄₅% increased to a peak faster and earlier with reperfusion at or before 3 hr than in the absence of reperfusion. Although both the rates of rise and decline of MM₄₅% were increased by early reperfusion, less variability was evident in the apparent rate of increase in MM₄₅% (figure 1, C). The decrease of MM₄₅% was delayed in some dogs, but was not associated with coronary stenosis or reocclusion at autopsy. The time to peak of MM₄₅% measured from the onset of coronary occlusion was reduced significantly with reperfusion at 1, 2, and 3 hr (table 1) compared with that in the nonreperfused control dogs (5.0 ± 1.1 hr, p < .05).

Rate of increase in MM₄₅%. The rate of increase in MM₄₅% in plasma was similar among dogs undergoing early reperfusion (table 1). Although absolute MM₄₅ and total CK activities in plasma varied over a considerable range, reflecting differences in infarct size, the rate of increase in MM₄₅% was influenced neither by the peak total CK activity (range 156 to 5648 IU/liter, r = −.1) nor the infarct size (range 0.9 to 38.4 g, r = −.003; table 1). The rate of increase in MM₄₅% in dogs

![FIGURE 2. MM₄₅% in dogs with persistent coronary occlusion (open circles; n = 9) and in dogs undergoing reperfusion 2 hr after coronary occlusion (closed circles; n = 6). Points represent mean ± SD.](http://circ.ahajournals.org/)}
undergoing reperfusion after 1, 2, or 3 hr of coronary occlusion (1.44 ± 0.42% min⁻¹, 1.28 ± 0.45% min⁻¹, 1.02 ± 0.27% min⁻¹, respectively) was markedly greater than that measured in dogs with persistent ischemia (0.29 ± 0.09% min⁻¹, p < .001; table 1 and figure 3). The rate of increase in MM₆₆% with reperfusion at 4 hr (0.48 ± 0.34% min⁻¹) was also greater than that in control dogs, but this difference was not statistically significant.

Discussion

These results show that the fraction of total CK activity in plasma contributed by the myocardial isoform MM₆₆ peaks very early after the onset of myocardial reperfusion. The rate of change in MM₆₆% in plasma after early reperfusion is consistent and markedly different from the rate in the absence of reperfusion, regardless of the magnitude of peak total CK activity or infarct size. Thus, analysis of changes in MM₆₆% in plasma provides an early and reliable index of myocardial reperfusion.

Conventional noninvasive criteria of reperfusion such as abrupt cessation of chest pain, rapid resolution of ST segment deviations, acceleration of Q wave development, and “reperfusion” arrhythmias,⁵ are neither sufficiently sensitive nor reliable for early and definitive detection of myocardial reperfusion. Chest pain may decrease because of many factors other than reperfusion, including reduction of anxiety or beneficial but indirect effects of concomitant pharmacologic therapy. Rapid resolution of ST segment changes or abrupt onset of ventricular arrhythmias occurs often after successful coronary recanalization, but the sensitivity and specificity of such criteria are limited.⁶,⁷

Reperfusion of ischemic myocardium accelerates the entry of myocardial enzymes released from necrotic cells into the circulating blood. Consequently, the rate of increase in CK activity in plasma and time of peak CK activity occur more rapidly after reperfusion than after persistent coronary occlusion.⁸,⁹ However, both depend on the interval between the onset of ischemia and reperfusion.¹⁰ Furthermore, neither total CK nor MB-CK activity peaks until 10 to 14 hr after the onset of chest pain in patients in whom reperfusion occurs within 7 hr.⁸,⁹ Thus, rapid confirmation of reperfusion in terms of absolute changes in total CK or MB-CK activities is not possible.

The difficulty in detecting reperfusion based on the time of occurrence of peak CK activity or the rate of increase in absolute CK activity in plasma is evident from our results. Considerable variation in CK time-activity curves was observed despite the similarities with respect to the location of the occlusive balloon on the circumflex coronary artery and the interval of ischemia before reperfusion within the groups of dogs (figure 1). Components contributing to this variability probably include differences in the extent of infarction (table 1) that are attributable, in part, to the heterogeneity of the coronary collateral circulation in dogs.¹¹

In contrast to the variability in absolute CK activity in plasma after reperfusion, the percentage represented by MM₆₆ exhibited consistent, time-dependent changes after reperfusion (figure 1). Our previous results in dogs with persistent coronary artery occlusion indicate that the consistency of isoform changes is related in part to the uniform kinetics of conversion of MM₆₆ to MM₆₈ and MM₈₆ to MM₈₆ isoforms and the uniform kinetics of elimination of each isoform from plasma despite differences in peak total CK activity and cumulative CK released after infarction.¹ Because MM₆₆% is the predominant isoform in canine myocardium and MM₆₆% in plasma from normal dogs is usually less than 20%,¹ an increase of plasma MM₆₆% is a sensitive index of recent release of enzyme from myocardium.

Three characteristics of the time-dependent change in MM₆₆% seem potentially useful as indexes of reperfusion: (1) the rate of increase in MM₆₆%, (2) time to peak of MM₆₆%, and (3) the rate of decrease in MM₆₆%. In this study, the time to peak of MM₆₆% occurred significantly earlier after reperfusion within 3 hr of the onset of ischemia than in the absence of reperfusion. The utility of this index of reperfusion is diminished, however, by the requisite time delay and frequent blood sampling necessary to establish that MM₆₆% has reached a peak. Determination of the rate of decrease in MM₆₆% entails an even longer delay. Moreover, the decrease in MM₆₆% was not consistent despite verified restoration of blood flow (figure 1, C). Inconsistency in the rate of decrease in MM₆₆% is compatible with continuing infarction, although coronary reocclusion was absent at autopsy. Nevertheless, the ambiguity likely to be encountered in detecting reperfusion in the face of such variations detracts from the potential value of the rate of decrease in MM₆₆% as an index of successful recanalization.

The rate of increase in MM₆₆%, however, appears to be a particularly useful index of myocardial reperfusion. Changes in this rate indicative of reperfusion were identifiable within 30 min after the onset of reperfusion. The rate was not influenced appreciably by either the timing of the onset of reperfusion within the interval of 1 to 3 hr or infarct size (figure 3; table 1). Reperfusion initiated within this interval was therefore readily distinguishable from persistent ischemia sim-
FIGURE 3. Rates of increase in \( \text{MM}_A \% \) (\( \text{MM}_A \% \min^{-1} \)) in dogs with persistent coronary occlusion and in dogs undergoing reperfusion 1, 2, 3, and 4 hr after coronary occlusion. *p < .001.

perhaps by the analysis of the rate of increase in \( \text{MM}_A \% \).

The rate of increase in \( \text{MM}_A \% \) after reperfusion was calculated with a linear function applied to the \( \text{MM}_A \% \) measured just before and 30 min after restoration of coronary blood flow. Based on the assumption that \( \text{MM}_A \% \) increased exponentially and reached a peak value within 30 min in most dogs, the linear rate of increase in \( \text{MM}_A \% \) probably underestimated the actual rate of release of \( \text{MM}_A \) from myocardium. Nevertheless, the significant differences between the linear rates of increase in \( \text{MM}_A \% \) in dogs undergoing reperfusion and nonreperfused control dogs clearly identified reperfusion based on a simple calculation requiring only two determinations of \( \text{MM}_A \% \).

The reliability of the rate of increase in \( \text{MM}_A \% \) as a marker of reperfusion is enhanced by its independence from infarct size (\( r = -0.003 \)). In contrast, both the rate of appearance of total CK activity and peak total CK activity were influenced markedly by infarct size (\( r = .98 \)). Consequently, a change in total CK activity is a less definitive criterion of reperfusion than the rate of increase in \( \text{MM}_A \% \).

The rate of increase in \( \text{MM}_A \% \) progressively declined as the duration of ischemia preceding reperfusion increased from 1 to 4 hr. This decline may relate to diminished release of \( \text{MM}_A \) from myocardium with late reperfusion. Decreased release of \( \text{MM}_A \) into blood could result from isofrom conversion or CK inactivation within necrotic myocardium and cardiac lymph. Insofar as clinical reperfusion by thrombolysis may effect a slower washout of CK from myocardium compared with the abrupt, complete restoration of coronary blood flow achieved in this study, the time in which the rate of increase of \( \text{MM}_A \% \) may be useful for detecting myocardial reperfusion in patients cannot be inferred from the present findings. However, acceleration of the appearance of total CK activity in plasma of patients after early reperfusion (i.e., within 3 to 4 hr of the onset of ischemia) suggests the likelihood of an analogous early time course of \( \text{MM}_A \% \) in plasma, permitting prompt detection of myocardial reperfusion.

We used chromatofocusing to separate MM-CK isoforms. The accuracy and precision of this nondenaturing procedure have been reported previously. With the modifications used, isofrom determinations could be completed in most plasma samples within 90 min after sampling. Continued refinement and development of new analytic methods should reduce the time required for analysis, thereby enhancing the clinical utility of the approach developed and facilitating rapid evaluation of the efficacy of interventions designed to restore blood flow to ischemic myocardium.

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