(54) Title: USE OF CREATINE KINASE AND ITS ISOZYME CK-MB AND HUMAN VENTRICULAR MYOSIN LIGHT CHAIN 1 IN THE DIAGNOSIS OF HEART FAILURE

(57) Abstract

In accordance with an aspect of the present invention methods for the early detection of a myocardial infarction and for the differentiation between a myocardial infarction and another cardiac event are provided. These methods comprise the sequential or simultaneous testing for creatine kinase and for human ventricular myosin light chain 1 in a sample selected from the group consisting of blood, serum and plasma.
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USE OF CREATINE KINASE AND ITS ISOZYME CK-MB AND HUMAN VENTRICULAR MYOSIN LIGHT CHAIN 1 IN THE DIAGNOSIS OF HEART FAILURE

FIELD OF THE INVENTION

This invention relates to a method which simultaneously assesses the presence or absence of two independent markers of myocardial damage to improve the accuracy of the early diagnosis of a myocardial infarction and for distinguishing between cardiac ischemic disease, such as angina, and myocardial infarction.

BACKGROUND OF THE INVENTION

Myosin is the major protein constituent of cardiac muscle and it consists of two myosin heavy chains and four myosin light chains. Two classes of light chains have been characterized in cardiac myosin: cardiac myosin light chain 1 with a molecular weight of 27,000 daltons and myosin light chain 2 with a molecular weight of 20,000 daltons.

The light chain of myosin is known to be released from cardiac muscle following myocardial infarction and may remain elevated in serum for several days [Khaw et al, CIRCULATION 58: 1130, (1978); Trahern et al., AM. J. CARDIOL 41: 641, (1978)]. Katus et al. [JACC, 2(3): 487] recently reported a correlation between the presence of myosin light chains and signs of ischaemia in the electrocardiogram and extent of coronary artery narrowing. It is also known that myosin light chain is released during ischaemia from other causes such as angina. An elevated plasma light chain concentration has also been observed in patients with congestive cardiomyopathy associated with an inflammatory infiltrate [Haver, E. J. MOL. & CELL CARDIOL., 17(Supplement 2): 53, (1985)].

There are a number of reports describing diagnostic methods for muscle diseases utilizing polyclonal or monoclonal antibodies directed at myosin light chains.
[Traherne et al. supra; Khaw et al. supra; Nagai et al., BIOCHEM. BIOPHYS. RES. COMMUN., 86: 683, (1979); Katus et al., AM. J. CARDIOL., 54: 964, (1984), Katus et al., MOL. IMMUNO., 19: 451, (1982); and Masahito et al. European Patent Application D205 177 A2). These diagnostic methods, however, suffer from a number of disadvantages such as cross-reactivity between cardiac myosin light chains as well as cross-reactivity with myosin light chains from other tissues (e.g. skeletal muscle) or other species.

It is known that cardiac myosin light chains have a distinct primary amino acid sequence which is different from the equivalent chains from skeletal or smooth muscle myosin. Patent applications for the DNA sequence of human ventricular myosin light chains 1 (HVMLC1) and 2 (HVMLC2) are pending in the United States [U.S. S.N. 241,672 (HVMLC2); U.S. S.N. 160,892 (HVMLC1)] and elsewhere. The identification of DNA sequences that code for these two human myosin light chains enables the preparation of highly specific monoclonal and polyclonal antibodies useful in diagnostic methods and for various types of therapy.

It is known that myocardial light chain (MLC) release follows a byphasic pattern [Hirayama, A. et al, JACC, 13 (2): 95A, (1989) "Byphasic Release of Cardiac Myosin Light Chains in Patients with Successful Thrombolysis in Acute Myocardial Infarction"]. According to Hirayama MLC is released early and rapidly approaches its peak concentration at 18 - 24 hours after the infarct. A second, subsequent release of MLC reaches its peak concentration at 3 - 5 days after the onset of the infarct.

Current serum assays depend on the leakage of intra-cellular enzymes, such as creatine kinase, or other proteins, from the damaged or necrotic cardiac cells which result from the myocardial infarction. Differentiation between myocardial ischaemia and unstable
angina or the detection of myocardial injury is often difficult. It is known that the release kinetics of cardiac myosin light chains differ from the release kinetics of creatine kinase and its isozymes around the time of acute myocardial infarction (AMI) [Katus, H.A., et al, AM. J. CARDIOL., 54: 964-970, (1984) "Diagnosis of Acute Myocardial Infarction by Detection of Circulating Cardiac Myosin Light Chains)].

The method disclosed by the present inventors is a method which surprisingly incorporates two assays, one of which is known. The second assay is novel in the extent of its specificity and sensitivity. The first, known, assay is the creatine kinase test which is the recognized standard test for the detection of a heart attack. This is the standard assay used by physicians today for the detection of heart attacks. CK, and its isozymes CK-MB and CK-MM are proteins that are known to leak out from the heart during a heart attack. Only the isoenzyme CK-MB shows unique specificity for the cardiac tissue in that it is not present in significant concentrations in extracardiac tissues. Unfortunately these proteins are not first detectable at meaningfully increased levels until 8 - 10 hours after the heart attack has occurred. Furthermore, these proteins are not detectable at peak concentrations until around 12 hours and, in some instances longer than 12 hours. It is currently standard practice to administer creatine kinase tests at three different times on a series of samples taken at the following times: the time of admission, 12 hours after admission, and 24 hours after admission before a final diagnosis is rendered. At the present time the CK assay is the best assay available for the detection and confirmation of the occurrence of a heart attack. Using this test alone a reliable positive diagnosis of a heart attack cannot be made without waiting for the full 24 hour period to pass.
There are other problems inherent in the known standard creatine kinase test assays aside from the delay, described above, in getting results which are diagnostically reliable. The most relevant problem, as related to the instant invention, is that the creatine kinase assay is not specific to the heart. Furthermore the test is useful only for the detection of a heart attack and is not diagnostic for other cardiac ischaemic events, such as angina. At the present time there are no known diagnostic assays which distinguish detection of heart disease from a heart attack.

DEFINITIONS

The terms creatine kinase (CK) and creatine phosphokinase are used interchangeably in this application. Both of these terms are intended to include the known isoymes of this enzyme. One skilled in the art would know when one or all of the creatine kinase enzymes would be appropriate.

HVLC1 and HVMLC1 are used interchangeably as abbreviations for Human Ventricular Myosin Light Chain 2. Similarly HVLC2 and HVMLC2 are used interchangeably as abbreviations for Human Ventricular Myosin Light Chain 2.

The terms heart attack, myocardial infarct and myocardial infarction are used interchangeably.

The terms myocardial ischaemic event and cardiac ischaemic event are used interchangeably.

Myocardial infarct is an example of one type of a myocardia ischaemic event. Myocardial ischaemic events other than a myocardial infarct include angina and other types of heart failure.

SUMMARY OF THE INVENTION

In accordance with an aspect of the present invention methods for the early detection of a myocardial infarction and for the differentiation between a myocardial infarction and another cardiac event are provided. These methods comprise the sequential or simultaneous testing for creatine kinase and for myosin
light chain in a sample selected from a tissue sample, serum sample, or other proteins containing sample.

In accordance with another aspect of the invention a method for the early detection of a myocardial infarction is provided. This method comprises:

(a) testing for creatine kinase in protein-containing sample;

(b) testing for myosin light chain in said protein-containing sample; and

(c) determining the presence or absence of creatine kinase and of myosin light chain in said sample.

In accordance with another aspect of the invention a method for differentiating between a myocardial infarction and an cardiac ischaemic event which is not a myocardial infarction is provided. This method comprises:

(a) testing for creatine kinase in a protein-containing sample;

(b) testing for myosin light chain in said protein-containing sample; and

(c) determining the presence or absence of creatine kinase and of myosin light chain in said sample.

In accordance with one aspect of this invention a method is described for the detection of myosin light chains in tissues, serum or other suitable sample of patients suspected of suffering from heart disease, heart failure or myocardial infarct.

In accordance with another aspect of the present invention a method is described for the simultaneous assessment of the presence or absence of two independent markers of myocardial damage, namely creatine kinase (CK) and its isozymes and human ventricular myosin light chain (HVMLC) to improve the accuracy of the detection and diagnosis of a myocardial infarction and, for distinguishing between cardiac ischemic disease, such as angina, and myocardial infarction.
In accordance with another aspect of the present invention an assay using antibodies prepared from known amino acid sequences of myosin light chain 1 is presented.

In accordance with a further aspect of the invention a highly specific assay is presented for the early detection of a myocardial infarct.

In accordance with another aspect of the invention an assay is presented for the differentiation and distinction between heart disease and a myocardial infarct.

In accordance with yet another aspect of the present invention diagnostic kits are described for the assays taught herein.

**BRIEF DESCRIPTION OF THE DRAWINGS**

Other and further advantages of the invention will be apparent to those skilled in the art from the following detailed description thereof, taken in conjunction with the accompanying drawings, in which:

Figure 1 is a graphic illustration of the mean serum levels of SGOT, CPK and HVMLC1 (HVLC-1) over time (hours) in 66 patients clinically diagnosed as suffering from an acute myocardial infarct (AMI);

Figure 2 is a graphic illustration of the mean serum levels of CPK and HVMLC1 (HVLC-1) over time (hours) in 86 patients identified as suffering from cardiac chest pain;

Figure 3 is a graphic illustration of the percent positive assays for elevated serum levels of CPK and HVMLC1 (HVLC-1) over time in 66 patients identified as having had an AMI;

Figure 4 is a graphic illustration of the percent positive assays for elevated serum levels of CPK and HVMLC1 (HVLC-1) over time in 86 patients identified as having had cardiac chest pain; the cut-off point for a positive result is 0.75 ng;
Figure 5 is the same as Figure 4 however a greater assay sensitivity is illustrated in Figure 5 in which the cut-off point for a positive result is 0.50 ng.

**DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS**

It is appreciated that the pain associated with angina pectoris which is due to ischaemia of the heart muscle, usually caused by coronary disease, is difficult to differentiate clinically from the pains associated with a myocardial infarct. It is shown in one aspect of the present invention that myosin light chain 1 and myosin light chain 2 are released as early as one hour after an ischaemic event such as acute myocardial infarct (AMI) or angina. In the instance of an AMI these myosin light chains continue to be released and are detectable in the blood stream of the patient for as long as 8 days. In the case of angina the continued release of the myosin light chains would not likely occur for such a length of time after the ischaemic event. CK, and its isozymes, is released only in the instance of an AMI. The kinetics of the release of these enzymes is seen in Figures 1 through 5.

In contrast the known creatine kinase assays do not reach the same level of release as the myosin light chains until 10 - 12 hours after the ischemic event, peaking at 24 hours. CK-MB levels peak rapidly, level off for 2 - 3 days after which they drop sharply. Creatine kinase proteins are not released unless there has been a myocardial infarct and would not be detected in other instances of heart disease such as angina pectoris.

The myosin light chain assay, of the present invention and the known creatine kinase assay are combined in the present invention to create a new assay for the detection of myocardial infarct. This combination assay simultaneously assesses the presence or absence of two independent markers of myocardial damage, namely CK (and its isozymes) and HVMLC. Such an assay is
not presently available and is particularly needed and useful for the detection of the early stages of a heart attack when creatine kinase protein levels have not yet risen to reliable diagnostic levels.

The novel combination assay of the present invention permits the early detection and recognition of a myocardial infarct thereby allowing the patient to receive appropriate treatment as quickly as possible. The combination assay shows a cumulative sensitivity approaching 100% accuracy in identifying whether or not an MI has occurred. The occurrence of a positive creatine kinase test and a positive HVMLC1 between 4 and 12 hours after the onset of a myocardial infarction permits an earlier and more accurate diagnosis than either test alone. Such a reliable diagnostic test, for this early phase of a heart attack, is not presently known.

While this combination of using the two assays is particularly useful in recognizing the early stages of a MI, further advantages are also appreciated.

One such surprising advantage is that by combining the two tests a novel method for differentiating between a heart attack and other ischaemic events causing cardiac pain, such as angina, is also provided. As seen in the data presented in example 1 the HVMLC1 assay of the preferred embodiment has a sensitivity of 97.6% and a specificity of 90% for myocardial ischaemia caused by any event, e.g. angina and myocardial infarctions. HVMLC1 is only 56% specific for acute myocardial infarctions because it is also released from the myocardium with reversible ischemic injury, e.g. angina. It has been shown that the positive biochemical diagnosis of a myocardial infarction with CK-MB testing is made with a sensitivity/accuracy of 77% by 12 hours and 96% within 24 hours [Lee and Goldman, Ann. Internal. Med., 106: 181–186, (1987)]. In comparison, the data presented in Example 1 illustrates that the combination of a positive
CK-MM or CK-MB and a positive HVMLC1 is 75% sensitive/accurate by 4 hours and 100% sensitive/accurate by 8 and 12 hours after the onset of a myocardial infarction in the positive identification of a MI. Based on these data the advantage of the combined assays to form a new methodology for the detection of myocardial infarction is clear. Surprisingly this data also illustrates additional advantage of the present invention. Namely, that the assay is diagnostic of heart disease, other than myocardial infarct, as well 12 to 24 hours a positive HVMLC1 assay and a negative CK, CK-MM or CK-MB assay (i.e. the combined assay) is indicative of heart disease other than a myocardial infarction. Support for these findings can be found in Figures 1 through 5.

Figure 1 illustrates the mean serum levels of the enzymes SGOT, CPK and of the myosin light chain HVMLC1 (referred to in the figure as HVLC-1) for patients clinically depicted as having AMI. Time from the initial chest pain is indicated on the x-axis (in hours). The mean serum level measured in the patient samples was divided by the normal serum level expected for that particular protein to generate the factors indicated on the y-axis of the Figure. For example, if the mean serum level for the protein was twice as high as the expected normal serum level of that protein then the datum was plotted on the y-axis at a factor of 2. If the sample level equalled the expected norm then the corresponding factor would be 1 and so on. Normal serum levels were always selected from the upper limit of the normal range.

It can be seen from the data plotted in Figure 1 that the HVLC-1 curve is consistently elevated from the onset of the initial chest pain. Two distinct peaks are seen in the HVLC-1 serum levels, an early peak at 8 to 10 hours and a later peak at 2 to 5 days. In contrast
the CPK enzyme levels remain at normal levels until six hours and do not peak until 12 to 24 hours after the onset of initial chest pain. The surprising elevation of the HVLC-1 levels at the early stages of the myocardial infarction permits a clear diagnosis of AMI as early as 7 or 8 hours after the initial onset of pain. This early detection, with nearly 100% certainty, of an AMI is not presently available through use of the standard CK enzyme assay alone.

The early and late elevated HVLC-1 levels provide other useful clinical information about the patient's health. Any elevation of HVLC-1 (and, as one skilled in the art would appreciate, presumably HVMLC2) is known to be diagnostic of an ischaemic event be it caused by a heart attack, angina or some other cardiac illness. Release of these myosin light chains is known to be associated with a cardiac ischaemic event. However, until the combined assay of the present invention, one has been able to appreciate the kinetics of the early release of this protein at the time of the ischaemic event since no known assay has been sufficiently sensitive and specific to reliably make such determinations. The use of known sequences of HVMLC1 and HVMLC2 now make this possible. The relevant DNA sequences and, by implication, amino acid sequences are described in the following list of copending patent applications: U.S. S.N. 160,892 and its c-i-p, U.S. S.N. 239,899, U.S. S.N. 241,672, CA S.N. 576,230 and EP S.N. 88312271.5.

Specific antibodies raised against one aspect of the present invention is its surprising specificity and sensitivity for human ventricular myosin light chains in the preferred embodiments described in Examples 1 and 2. The surprising sensitivity and specificity of the present assay likely results from the use of the known DNA sequences which can be used to synthetically prepare the HVMLC1 and HVMLC2 protein molecules. These, in turn, are
used as antigens, to generate the specific antibodies used in the immunoassay measurements. The use of these antibodies of unique specificity significantly reduces, if not eliminates, the high incidence of cross-reactivity one would otherwise expect. This, in turn, now makes it possible to assay and observe the enzyme kinetics associated with ischaemic events. This was not previously possible.

The data presented in Figures 1 and 2 illustrates how a test combining the creatine kinase assay and the HVMLC assay successfully differentiates between pain associated with AMI and pain resulting from unstable angina or subendocardial MI. In Figure 1 data is presented for those patients who were monitored prior to the onset of the AMI. At time less than zero hours the angina which the patient is experiencing results in elevated HVLC-1 levels but CPK levels remain unchanged. If by 8-10 hours no elevation in CPK occurs then it is likely that no heart attack has occurred (it is recognized that occasional extremes in variability can occur between individuals). If, on the other hand, by 8 hours elevated CPK is detected then it can be said with nearly 100% certainty that the patient has suffered from an AMI. The peaks of the mean serum levels for CK are noticeably reduced when the non-AMI patients are averaged into the sample (figure 2). This is because no CK response is observed in patients suffering from cardiac chest pain not related to an AMI. As expected, a similar reduction in mean serum level of HVLC-1 is not observed.

Figures 3, 4 and 5 graphically illustrate the sensitivity of the HVLC-1 assay. The percentage of patients proven to have had a heart attack who also had positive results with the relevant test are presented on a scale of 0% to 100% along the y-axis. In other words the number of true positives as ascertained by the test data is divided by the number of patients known to have had the disease and multiplied by 100 to get the "percent
positive*. In Figures 3 and 4 true positives were assessed as values above 0.75 ng and in Figure 5 the cut-off value was slightly lower at 0.50 ng. Selection of the cut-off value will vary depending on the minimum sensitivity achieved during that test run. One skilled in the art would know how to make this assessment.

In the present invention a method is described to improve the accuracy of the early diagnosis of a myocardial infarction by testing simultaneously for two independent markers of myocardial damage. In one preferred embodiment this method comprises the use of detecting simultaneously the presence of CK-MM or CK-MB using known creatine kinase assay techniques and the presence of human ventricular light chain-1 (HVMLCl) using solid-phase enzyme immunoassays. One skilled in the art would know other known suitable immunoassay technologies which could also be used.

One advantage of enzyme immunoassays, such as the one disclosed for the detection of HVMLCl in the preferred embodiment of this invention (Example 2), is the potential use of colour production for the ready detection of a positive assay. Using different enzyme substrate combinations the presence or absence of a positive CK assay and/or a positive HVMLCl assay will be readily apparent based on the presence or absence of the colours produced. For example, it is known that the alkaline phosphatase enzyme, in conjunction with the para-nitrophenyl substrate, will produce a reaction product having a yellow colour. In contrast, it is known that substrates used in conjunction with the beta-galactosidase enzyme will produce reaction products having a blue colour. The use of distinctive colour reactions allow the combination of these two assays to distinguish between the CK assay and the HVMLCl assay thereby facilitating quick and ready detection of the presence or absence of myosin light chains and CK-MB or CK-MM in the patient's blood by merely assessing colour
change. In one preferred embodiment of the present invention the two assays would be performed on a nitrocellulose membrane such that when both of the assays yield a positive result a green colour (yellow + blue) occurs since both assays can be performed on the same nitrocellular dish.

In one preferred embodiment the presence of CK or CK-MB is detected in one location on a suitable solid phase membrane such as nitrocellulose and the presence of human ventricular light chain-1 (HVMLC1) is assayed at another location on the same membrane with the two assays overlapping in one common area [SHOULD THIS BE ILLUSTRATED?]. The two separate solid-phase enzyme immunoassays could be processed simultaneously as one single combined diagnostic test. The use of other suitable solid-phase supports for such immunoassays would be known by one skilled in the art.

The capture antibody for the CK assay could be either a polyclonal or a monoclonal antibody which recognizes CK-MM and/or CK-MB. These capture antibodies could be bound, either covalently or non-covalently, to the solid phase. In similar fashion, the HVMLC1 assay could utilize sequence specific polyclonal or monoclonal antibodies which recognize HVMLC1. These too could be either covalently or non-covalently bound to the solid phase as capture antibody for HVMLC1. Antibodies raised against either the natural, native proteins or the synthetic "DNA sequenced" proteins could be used.

When a blood, plasma or serum sample is brought in contact with the solid phase support and the capture antibodies bound thereto specific antigens, if these antigens are present, in the blood, plasma or serum of the patient being tested will bind to the capture antibodies. Antigen specific polyclonal or monoclonal detection antibodies bearing suitable enzyme labels, or suitable control detection antibodies, are then brought in contact with the solid phase support. Detection
antibody specific for CK-MM or CK-MB will bind to the CK-MM or CK-MB antigen if it is present on the solid phase support. Similarly, the detection antibodies specific for HVMLC1 would bind to any HVMLC1 antigen which is bound to the capture antibodies.

Unbound detection antibodies are removed with a washing solution. A colour development solution containing the appropriate substrate for either or both enzymes bound to the detection antibodies is then added to the membranes once the detection antibodies have bound to their appropriate antigens, thereby indicating the positive presence of these antigens in the patient's plasma or serum or blood when the substrate is converted to its coloured end product after reacting with the bound enzyme. Where no such antigens are present then no detection antibodies will be bound and no enzyme will be present to react with the substrate in the colour development solution. The amount of colour formed will be directly proportional to the amount of antibody-enzyme that is bound to CK-MM, CK-MB or to HVMLC1 on the membrane thereby indicating the proportion of these antigens in the sample. If the test is set up such that substrate reaction products having different colours are used for the different antigens then ready detection of positive results will be possible. When performed on nitrocellulose the tests can be overlapped such that a third colour is produced when both test results are positive.

Other enzyme immunoassays (enzyme based, radioactive based, fluorescence based, or otherwise) could be used for detecting the presence or absence of relevant antigens and these would be known to one skilled in the art. One skilled in the art would also know that human ventricular myosin light chain 2 could be used in a similar manner as described herein for HVMLC1. One skilled in the art would also appreciate that samples
other than blood, serum or plasma could also be tested as described herein.

Further details of the preferred embodiments of the invention will be understood from the following examples which are understood to be non-limiting with respect to the scope of the invention.

EXAMPLE 1 - Combination Testing for Detection and Diagnosis of Heart Attack and Other Cardiac Pain Using CK Assay and HVMCL1 Assay

116 patients were assessed upon admission to emergency in hospital. All patients were tested using solid-phase enzyme immunoassays (see Example 2) for the presence of CK or CK-MB and for the presence of myosin light chain 1 (HVMCL1) in the serum of patient samples.

Patients tested were also assessed based on clinical presentation and were broken down into three groups:

1. 66 patients judged to have acute transmural myocardial infarct (AMI);

2. 20 patients with unstable angina or subendocardial M.I.; and

3. 30 patients with no evidence of ischaemia or infarction and judged to have non-cardiac pain or other diagnosis.

Clinical criteria for assessing cardiac chest pain included:

a) definite M.I. on electrocardiogram (ECG) as measured by ST elevation, QE wave evolution or deep T neg (subendo);

b) evidence of ischaemia (ST depression > 1mm, 2 leads);

c) typical chest pain of greater than 30 min. duration;

d) angiographic evidence of coronary artery disease; and
e) elevation of CK, CK-MB or SGOT with typical curve in absence of another cause.

Patients were assessed as having no cardiac pain by the following criteria:

a) no ECG evidence of M.I. or ischaemia;
b) no elevation of CK, CK-MB or SGOT enzymes, or elevated enzymes explained by documented non-cardia cause, or angiography negative for coronary artery disease; and
c) no evidence on GXT or thallium scan (if done).

Table 1 details the findings of the myosin light chain (MLC) HVMLC1 immunoassays. The sensitivity, specificity and predictive value of the HVMLC1 assay tests are summarized in Table 2. The data generated in these studies is presented graphically in Figures 1 through 5.
### TABLE 1

#### 86 Patients with "Cardiac Chest Pain"

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<th>TIME</th>
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#### 66 Patients with Acute M.I.

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#### 50 Patients with No M.I. (Non-Cardiac & Angina)

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<tr>
<td>22/50</td>
<td>28/50</td>
</tr>
</tbody>
</table>

<sup>1</sup>NORMAL MLC < 0.6 ng/ml.
<table>
<thead>
<tr>
<th></th>
<th>ACUTE MI (4 days)</th>
<th>CARDIAC PAIN MI &amp; ANGINA</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Sensitivity</strong></td>
<td>98.5%</td>
<td>97.6%</td>
</tr>
<tr>
<td><strong>Specificity</strong></td>
<td>56%</td>
<td>90%</td>
</tr>
<tr>
<td><strong>Predictive Value</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Positive Test</td>
<td>75%</td>
<td>96.6%</td>
</tr>
<tr>
<td>Negative Test</td>
<td>96%</td>
<td>93%</td>
</tr>
</tbody>
</table>

'MLC immunoassay refers to the HVLMC1 immunoassay described in this specification.'
EXAMPLE 2 - Sandwich ELISA for detection of HVMLCl

1. A 96-well microtiter plate was coated with 0.1 ml of "159-3" antibody (20 ug/ml). Coating was done in 0.05M carbonate buffer pH9.6.

2. The plate was covered with adhesive cover and incubated overnight at 4°C.

3. The coating solution was removed by shaking out contents of the wells into a sink and blocking each well with 0.2 ml of diluting buffer.

4. The wells were emptied and patted dry on an absorbent paper.

5. 0.1 ml of each control (standard curve) and sample were added into wells. The plate was covered with an adhesive cover and incubated at 37°C for 1 hour.

6. The contents were shaken out of the wells. Using a squeeze bottle the wells were washed 3 times with wash buffer. The wells were dried on an absorbent paper between washes.

7. 0.1 ml of "504" antibody (1 ug/ml in diluting buffer) was added. The plates were covered with an adhesive cover and incubated at 37°C for 30 min.

8. Step 6 was repeated.

9. 0.1 ml of goat anti-rabbit horseradish peroxidase-conjugate was added at 1:2000 dilution in diluting buffer. The plate was covered with an adhesive cover and incubated at 37°C for 30 min.

10. Step 6 was repeated.
11. 0.1 ml of peroxidase substrate (ortho-phenylenediamine) solution was added and incubated at room temperature for 15 min.

12. The reaction was stopped by adding 0.1 ml of IN H$_2$SO$_4$.

13. The absorbance was read at 490 nm using a microtiter plate spectrophotometer.

The diluting buffer referred to above was 0.005% polyoxyethylene sorbitan monolaureate (TWEEN 20), 0.25% BSA, in phosphate - buffered saline, pH 7.4 (approx.).

The wash buffer used was 0.05% TWEEN 20 in phosphate - buffered saline, pH 7.4 (approx.).

The antibodies identified as "159-3" and "504" were antibodies having specificities for particular sequences unique to HVMLC1.
21

CLAIMS

1. A method for the early detection of a myocardial infarction comprising the steps described herein with reference to the accompanying figures.

2. A method for differentiating between a myocardial infarction and a cardiac ischaemic event which is not a myocardial infarction comprising the steps described herein with reference to the accompanying figures.

3. A method for the early detection of a myocardial infarction comprising the sequential or simultaneous testing for creatine kinase and for myosin light chain in a tissue or serum sample.

4. A method for differentiating between a myocardial infarction and a cardiac ischaemic event which is not a myocardial infarction comprising the sequential or simultaneous testing for creatine kinase and for myosin light chain in a tissue or serum sample.

5. A method for the early detection of a myocardial infarction comprising:
   (a) testing for creatine kinase in a protein-containing sample;
   (b) testing for myosin light chain in said protein-containing sample; and
   (c) determining the presence or absence of creatine kinase and of myosin light chain in said sample.

6. A method for differentiating between a myocardial infarction and a cardiac ischaemic event which is not a myocardial infarction, said method comprising:
   (a) testing for creatine kinase in a protein-containing sample;
(b) testing for myosin light chain in said protein-containing sample; and
(c) determining the presence or absence of creatine kinase and of myosin light chain in said sample.

7. A method of claim 5 or 6 wherein said sample is one of a series of samples taken from a patient at different times after a cardiac ischaemic event has occurred.

8. A method of claim 7 wherein said sample is taken between eight to ten hours after a cardiac ischaemic event has occurred.

9. A method of claim 8 wherein said sample is a sample selected from the group consisting of human blood, human serum and human plasma.

10. A method of claim 9 wherein said myosin light chain is human ventricular myosin light chain 1.

11. A method of claim 10 wherein said creatine kinase test and said myosin light chain test are performed sequentially on said sample.

12. A method of claim 10 wherein said creatin kinase test and said myosin light chain test are performed simultaneously on said sample.

13. A method of claim 11 or 12 wherein said creatine kinase test and said myosin light chain test are enzyme immunoassays performed on nitrocellulose paper.

14. A method of claim 13 wherein said creatine kinase immunoassay and said myosin light chain immunoassay are executed as two spots, on the same nitrocellulose paper and the enzyme-substrate combination used in the creatine kinase immunoassay differs from the enzyme-substrate
combination used in the myosin light chain immunoassay such that the coloured reaction products of each immunoassay are different colours.

15. A method of claim 14 wherein said spots overlap.

16. A method for the detection of myosin light chains in blood, serum or plasma comprising the steps of:
   (a) creating a myosin light chain specific antibody using one or more portions of a synthetically sequenced myosin light chain protein; and
   (b) using said antibody in an immunoassay to detect the presence or absence of myosin light chain antigen in blood, serum or plasma.


19. A method of claims 16, 17 or 18 wherein said antibody is a monoclonal antibody.

20. A kit comprising the materials required to perform the method of claim 14 or 15.
MEAN SERUM LEVELS - AMI
86 PATIENTS

FIG. 1.

MEAN SERUM LEVELS - ALL CHEST PAINS
86 CARDIAC PATIENTS

FIG. 2.

SUBSTITUTE SHEET
2/3
SENSIVITY - AMI (ECG or CK POSITIVE)

86 PATIENTS

FIG. 3.

SENSITIVITY - ALL CARDIAC CHEST PAINS
86 PATIENTS

FIG. 4.

SUBSTITUTE SHEET
3/3

SENSITIVITY-ALL CARDIAC CHEST PAINS

86 PATIENTS

% POSITIVE (≥ 0.5 Hg)

TIME FROM INITIAL CHEST PAIN (HOURS)

+ CPK  ○ HVLC-1

FIG.5.
INTERNATIONAL SEARCH REPORT

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC: G 01 N 33/548, G 01 N 33/577

II. FIELDS SEARCHED

Classification System Classification Symbols

IPC G 01 N, C 12 Q

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched

III. DOCUMENTS CONSIDERED TO BE RELEVANT

<table>
<thead>
<tr>
<th>Category</th>
<th>Citation of Document, with indication, where appropriate, of the relevant passages</th>
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<tbody>
<tr>
<td>Y</td>
<td>Scand. J. Clin. Lab. Invest., volume 44, no. 8, 1984, O. Baadsgaard et al.: &quot;Myoglobin concentration, creatine kinase, and creatine kinase sub-unit B activity in serum after myocardial ischaemia&quot;, pages 679-682 see the whole article</td>
<td>1-20</td>
</tr>
<tr>
<td>A</td>
<td>Biological Abstracts; volume 85, no. 5, 1988, (Philadelphia, PA, US), M. Isobe et al.: &quot;Quantitative relationship between left ventricular function and serum cardiac myosin&quot;</td>
<td>1-20</td>
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</tbody>
</table>

* Special categories of cited documents: A
  - A* document defining the general state of the art which is not considered to be of particular relevance
  - A" earlier document but published on or after the international filing date
  - A" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
  - A" document referring to an oral disclosure, use, exhibition or other means
  - A" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"A" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search: 4th September 1990
Date of Mailing of this International Search Report: 1. 10. 90

International Searching Authority: EUROPEAN PATENT OFFICE

Signature of Authorized Officer: M. PEIS
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