Early Diagnosis of Acute Myocardial Infarction Using Immunosensors and Immunotests

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Various biochemical markers are available to detect cardiac tissue injury after an acute myocardial infarction (AMI) and to estimate the extent of this injury. One of the markers used to detect AMI early after onset of symptoms is heart-type fatty acid-binding protein (H-FABP). To make it clinically applicable it is important to develop rapid diagnostic sensors to assess its concentration. Due to the requirement of skilled technicians and lengthy assay procedures, most immunochemical assays for H-FABP are of limited use for routine clinical practice. A rapid and quantitative immunotest for the detection of H-FABP in serum samples has been successfully developed with a performance time of 10 min. Fifty-one serum samples from patients were evaluated using a conventional ELISA and the newly developed test. A good correlation was found with $r^2 = 0.9585$. The detection limit of the test was 2.8 µg/L. For the pooled serum sample with a lower H-FABP concentration (4 µg/L), the calculated intra-assay coefficient of variation (CV) was 10%, and the inter-assay CV was 15%; for the pooled serum sample with a high H-FABP concentration (103 µg/L), the intra-assay CV was 8% and the inter-assay CV was 10%. Furthermore, the immunotest can be stored either at 4°C and room temperature for up to one year without significant loss of activity. Finally, a one-step FABP test so-called CardioDetect® which was derived from the serum immunotest has been designed for qualitative determination of H-FABP in whole blood samples. It requires no sample pretreatment and gives result within 15 min. Thirty-nine patients presenting with chest pain and suspected AMI were studied. Using an upper reference level of 7 µg/L, the specificity of the rapid test was 94%. Both sensitivity and negative predictive value (NPV) were 100% implying that 100% of nonAMI patients can be excluded with no false negative results. With this rapid and sensitive immunotest, H-FABP will be soon introduced to clinical practice.

Key Words: Heart-type fatty acid-binding protein; Enzyme-linked immunosorbsent assay; Acute myocardial infarction; Immunosensor.

INTRODUCTION

In the 2000 World Health Report, the World Health Organization (WHO) recognized the 10 leading killer diseases worldwide. Coronary heart disease (CHD) was ranked number one and acute myocardial infarction (AMI) accounted for the greatest percentages of deaths from
Despite decades of investigation into the diagnosis of AMI, the diagnostic process is still quite complex because the majority of patients with chest pain fall in the low or medium risk category with atypical symptoms and nonspecific electrocardiographic (ECG) changes. It has been reported that each year up to 70% of patients admitted to coronary care units for suspected AMI are later discharged with a different diagnosis costing billions of dollars.

The WHO’s criteria for diagnosing AMI were developed in 1979 and specify that two of the following three criteria must be met: (1) characteristic chest pain (usually more than 20 min); (2) diagnostic ECG changes; and (3) unequivocal elevation of cardiac enzymes. Many of the biochemical markers that came into use after the publishing of this criteria are not themselves enzymes, such as myoglobin and the troponins. This has lead to a call for revision of the criteria. Recently redefined criteria have been introduced that make specific recommendations on the use of biochemical markers of myocardial necrosis, especially the cardiac troponins, to diagnose myocardial infarction. Serial measurement of biochemical markers is now universally accepted as an important determinant in AMI diagnosis. However, the early diagnosis of AMI is still problematic in those patients without obvious ST-segment elevation because cardiac troponins are not detected frequently until after 6 h and a repeated measurement is necessary at 8–12 h after admission. Prompt diagnosis and treatment increase the survival rate, and the rapid exclusion of myocardial infarction can help to reduce unnecessary stays in intensive care units. Therefore, a rapid sensor for early diagnosis of AMI is crucial.

PHYSIOLOGICAL BACKGROUND

Biochemical markers are of increasing importance in diagnostic strategies for ruling in and ruling out AMI, particularly when electrocardiographic findings do not allow a diagnosis. Today, cardiac markers are a multimillion-dollar industry. In the Western world, serial testing for cardiac markers are performed on nearly every patient presenting with chest pain. The biochemical markers that are currently used by physicians to aid in the diagnosis of AMI are myoglobin, creatine phosphokinase isoenzyme MB (CK-MB), cardiac troponin I (cTnI), and cardiac troponin T (cTnT). The differences in the time that it takes each marker to reach peak concentration has made it standard practice for clinicians to make use of at least two different markers in tandem; an early marker, and a later one (Table 1).
The differences among the various cardiac proteins in the time at which they appear in plasma after AMI relate to the molecular size of the proteins. Smaller proteins generally appear in plasma more rapidly than larger proteins. Myoglobin is a 17.8 kDa oxygen binding heme protein present in both cardiac and skeletal muscle. As a small molecule, myoglobin diffuses quickly into the circulatory system from the areas of ischemic injury (Table 1). However, as it is not cardiac specific, elevated levels should be interpreted with caution if the patient being evaluated has renal dysfunction or skeletal muscle injury.\[9\]

Heart-type fatty acid-binding protein (H-FABP) is a small (MW 15 kDa) cytoplasmic protein that is abundant in cardiomyocytes. It is released from the heart early after the onset of infarct (Table 1), whereafter its plasma concentration increases manifold. Several studies indicate the usefulness of the plasma H-FABP concentration as an early biochemical marker for myocardial infarction diagnosis.\[10–12\]

Heart and skeletal muscles contain the same type of H-FABP,\[13,14\] but its concentration in the heart is several fold higher than that in the skeletal muscles.\[10\] The concentration of H-FABP in the plasma of healthy persons is relatively low (2–6 μg/L) making FABP a diagnostic marker with high sensitivity.\[15\] Ishii et al.\[16\] showed that H-FABP is a more sensitive and specific marker than myoglobin for the early diagnosis of AMI within 6 h, particularly within 3 h, of the onset of chest pain. It was reported that perioperative myocardial injury can be diagnosed from the release of cardiac markers at 30 min after the start of reperfusion, and H-FABP is a more suitable marker than CK-MB or myoglobin for early assessment of post-operative myocardial infarction.\[17\] FABP is also quickly eliminated from the blood stream by renal clearance, which makes it a useful marker for identifying recurrent infarction. However, in patients with chronic renal failure the preinfarct plasma concentration of FABP is very likely to be high and elevates over a longer period of time.\[18–20\] Thus, caution must be taken when using FABP for early

<table>
<thead>
<tr>
<th>Marker</th>
<th>Molecular weight (kDa)</th>
<th>Initial elevation (h)</th>
<th>Time to peak (h)</th>
<th>Return to normal (h)</th>
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<td>1–3</td>
<td>6–8</td>
<td>24</td>
</tr>
<tr>
<td>Myoglobin[7,8]</td>
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<td>1–3</td>
<td>5–8</td>
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<tr>
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<td>3–6</td>
<td>14–18</td>
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<tr>
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<td>3–6</td>
<td>10–12 days</td>
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<tr>
<td>CK-MB[5–8]</td>
<td>86</td>
<td>3–8</td>
<td>9–24</td>
<td>48–72</td>
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</tbody>
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diagnosis of myocardial infarction in cases of renal insufficiency and skeletal muscle injury.\textsuperscript{[10,21]}

\textbf{THE DEVELOPMENT OF FABP IMMUNOASSAYS}

\textbf{Sandwich Enzyme-Linked Immunosorbent Assay (ELISA)}

Sandwich ELISA for FABP has been successfully developed in Japan in 1995 and in Professor Jan F. C. Glatz's group in Maastricht in 1997 with the total performance times of 4 h and 45 min, respectively.\textsuperscript{[15,22]} A one-step sandwich ELISA for plasma FABP described by Wodzig et al.\textsuperscript{[15]} uses two monoclonal antibodies as catcher and detector antibodies with horseradish peroxidase as an enzyme label. The detection limit of the assay was 0.2 \( \mu \)g/L and a coefficient of variation of less than 10\% in inter- and intra-assay was reported.

\textbf{Electrochemical Immunosensors}

\textbf{The First FABP Sensor}

An electrochemical immunosensor for FABP was initially reported by the Muenster immunosensor group [Fraunhofer Institut fuer Chemische und Biosensorik (ICB)] in 1996.\textsuperscript{[23]} Using bovine FABP as a model, two approaches were developed based on (1) a competitive immunoassay, in which defined amounts of glucose oxidase (GOD)-labeled FABP competed with free FABP in sample to bind to immobilized capture antibodies; (2) a sandwich immunoassay, in which free FABP bound to immobilized capture antibodies and formed a sandwich with secondary GOD-labeled antibodies (Fig. 1). In both approaches, a resultant decrease in oxygen concentration after adding glucose was determined using an amperometric oxygen electrode at \(-600\) mV vs. Ag/AgCl.

The sandwich principle for determining human FABP by using monoclonal anti-bovine FABP antibodies as the capture and GOD-labeled polyclonal goat-anti-human FABP antibodies as the detector provided far superior results. The monoclonal antibodies were immobilized on a preactivated “Immunodyne” membrane, which was mounted on a Clark-type oxygen electrode. The sensor was operated with a single use of the membrane. In clinical application, the regeneration technique could not be used due to contamination risks.
The calibration curve was linear \((r = 0.98)\) within the range 5–80 \(\mu\)g/L. Recovery experiments using plasma spiked with purified human FABP yielded an average recovery of 93 ± 5\%, which compared well with the recovery of the sandwich ELISA (93.5\%).\(^{[23]}\) Thus, the development of this enzyme immunosensor made the measurement of human FABP in plasma samples of cardiac patients feasible for the first time.

Although a much shorter assay time was achieved (about 30 min), the preparation of the sensor was very laborious. Moreover, because all of the samples had to be diluted with the buffer at least 10 times, the overall sensitivity was not sufficient to distinguish patients with micro-necrosis.

The EUROCARDI Trial and the FABP Sensor

The development of a FABP immunosensor has been greatly propelled by a European Union project “EUROCARDI” which took place during 1994–1996 under the leadership of Jan F. C. Glatz’s group in Maastricht.\(^{[24]}\) As depicted in Fig. 2, a sandwich assay using an alkaline phosphatase (ALP) label was exploited to develop on amperometric immunosensor using screen printed graphite sensors.
The antibody-coated working electrode stored at 4°C was stable for at least three months. A recovery test was performed using plasma spiked with eight different concentrations of recombinant FABP in the range of 0–350 μg/L. The mean values calculated from eight measurements of each sample revealed recovery rates between 80 and 100%. To determine the assay precision, four patient samples with different FABP concentrations ranging from 0–300 μg/L FABP, as assessed by ELISA, were measured eight times. The intra- and inter-assay coefficients of variation were between 10 to 15% and 9 to 16%, respectively. Six plasma samples, taken from a patient during the first 24 h after AMI, were measured by the ELISA described by Wodzig et al.\cite{15} and the immunosensor. The correlation between these two methods was quite well.

FABP measurement for plasma using the immunosensor has been feasible. However, it required several working steps (incubation and washing). The entire measurement was also restricted to a quite complicated semiautomatic device and needed at least 20 min to complete. Moreover, this sensor could be used only by well-trained personnel.

**Figure 2.** Principle of the amperometric EUROCARDI immunosensor using a screen-printed graphite working electrode.\cite{24}
Furthermore, the sensitivity of the immunosensor was not sufficient to distinguish patients with micro-necrosis.

Nonseparation Thick Film Immunosensor

To develop nonseparation electrochemical sensors, all interferences from the real sample should be eliminated. This can be done with novel mediator-modified redox electrodes (working free of interferences near 0 mV vs. Ag/AgCl) using imprinted polymer thick-film pastes. The working electrode can be combined together with reference and auxiliary electrodes on a plastic chip. The working electrode can also be printed as a microelectrode array (similar to thin-film technology). Therefore, high current densities can be obtained. The antibodies can be directly immobilized on the working electrode, as in the case of the screen printed immunosensor.

In 1995, a nonseparation washless thick-film immunosensor was developed by Calum McNeil’s group in Newcastle-upon-Tyne. A screen printed electrode with immobilized capture antibodies and horse-radish peroxidase (HRP) as an indicator enzyme was exploited. HRP detected hydrogen peroxide (H$_2$O$_2$), which was generated from a special substrate (5-bromo-4-chloro-3-indolyl phosphate, BCIP) by ALP, with high sensitivity. H$_2$O$_2$ could be detected only if it was generated near an electrode surface (Fig. 3). Catalase was added to destroy H$_2$O$_2$ in the bulk generated by unbound detector antibodies labeled with ALP to prevent interference.

In another format, an enzyme channeling immunoassay has been successfully developed to measure FABP in Judith Rishpon’s group in Tel Aviv, in collaboration with our group at HKUST and Professor Jan F. C. Glatz’s group in Maastricht. GOD generated H$_2$O$_2$ at a sensor surface after addition of glucose. HRP converted iodide to iodine only if secondary HRP-labeled antibodies formed a sandwich with FABP and capture antibodies (Fig. 4).

On-Line Displacement Flow Immunoassay

An on-line displacement flow immunoassay has been developed in our group at HKUST in collaboration with the University of Groningen in the Netherlands. Preliminary data showed that the system could be able to continuously monitor on-line an increase of FABP in buffer solution.
In a standard displacement flow immunoassay, analytes in a sample create active dissociations of labeled antigens (or antigen homologues) from antigen binding sites of immobilized antibodies, where after labeled substances are measured. Such systems have been described for molecules.

**Figure 3.** Schematic diagram of a nonseparation thick-film immunosensor.[25]

**Figure 4.** Principle of a nonseparation amperometric enzyme channelling immunoassay with immobilized GOD and capture antibody on the surface of a polyethyleneimine (PEI) modified graphite electrode.[26]

In a standard displacement flow immunoassay, analytes in a sample create active dissociations of labeled antigens (or antigen homologues) from antigen binding sites of immobilized antibodies, where after labeled substances are measured. Such systems have been described for molecules...
up to 1 kDa (mainly pesticides and drugs). It was the first time in literature to demonstrate a displacement for detection of a small protein, H-FABP (15 kDa). The system applies an inverse set-up: enzyme-labeled monoclonal antibodies are associated to FABP immobilized on N-hydroxysuccinimide (NHS) activated Sepharose and displaced by FABP in sample. It allows detection of both physiological (2–12 µg/L) and pathological concentrations (12–2000 µg/L) of FABP in an on-line flow system. Lysozyme, a protein of similar size to FABP, did not show any replacement.

To further investigate the principles underlying the displacement assay of FABP in plasma samples, continuous measurement of FABP was mimicked by repeated addition of FABP containing solutions followed by several washing steps. In the presence of free FABP, the enzyme-labeled antibodies dissociated and were subsequently quantified. Significant displacement in the presence of free FABP was observed in both buffer and human plasma for duration of at least 9 h. Combined with a slow flow on-line sampling techniques such as microdialysis or ultrafiltration, the continuous signal of the flow displacement immunoassay can be used to its full potential.

**Immu-Affinity Filtration Chromatography**

Immu-affinity filtration chromatography has been successfully developed to detect FABP by Albert Chu at EY Laboratories (Hong Kong and San Mateo, California), our group at HKUST and Professor Jan F. C. Glatz’s group at Cardiovascular Research Institute Maastricht (CARIM, Maastricht, The Netherlands). The assay has used colloidal gold as a label which gives an immediate signal instead of a time-dependent reaction with enzyme.

With the immuno-affinity filtration chromatography approach, a configuration is vertically designed in a way that speeds up antibody–antigen binding kinetics. Capture antibodies are bound at the surface of a chemically modified cellulose pad in a sophisticated way to avoid unspecific binding of detector antibodies. The deeper cellulose layers suck the fluid of a sample through within 1 min. First of all a drop of the sample is added to the surface and sucked immediately in. Then, colloidal gold-labeled antibodies are added. A washing step is required to remove all unbound or nonspecifically bound colloidal gold-labeled antibodies.

The FABP assay is based on a sandwich principle, in which a mixture of goat polyclonal and mouse monoclonal capture antibodies are immobilized on the nitrocellulose (NC) membrane and a colloidal
gold-labeled monoclonal antibody is used as detector. If a sample has an FABP concentration in the physiological range (below 10 μg/L), no red spot can be clearly visualized. However, a red spot can be observed if the sample has an FABP concentration over the physiological range. It gives easily interpreted results within minutes. The results can be qualified as a "Yes/No" result or even be quantified by measuring the intensity of a red spot using a Digital Image Analyzer.

In Maastricht 242 serial blood samples obtained from 83 patients admitted to the hospital with chest pain (72 AMI, 9 unstable angina, 2 other causes) were studied and the test was validated against sandwich FABP ELISA. Using an upper reference level of 12 μg/L (83 samples below, 159 samples above the cut-off), the specificity of the rapid test was 96% and the sensitivity 86%.

Limitations of the Developed Immunoassays

The immunoassays described above offer practical ways to assess FABP concentration in serum or plasma samples. However, they are restricted to rather complicated semiautomatic laboratory devices and take at least 30 min for the immunosensor and 45 min for the ELISA to complete. In addition, they require various incubation and washing steps; therefore, only a well-trained person can perform those assays. Because of the urgency of an infarction situation, ELISA and immuno sensors cannot offer a convincing test for rapid AMI diagnosis.

Furthermore, the immuno sensors reported generally have two drawbacks due to interferences and electrode fouling when applied to real sample analysis. An excellent specificity of a biochemical system is seriously compromised by a partial selectivity of an electrode. In other words, the electrode will oxidize not only the products produced by an enzymatic reaction but also other species present in solution (such as ascorbic acid, uric acid etc.), which results in a larger current response and thus leads to an over-estimation. Complex samples such as serum contain enormous amounts of inter ferents. In addition, the adsorption of nonelectroactive species on electrode surface referred as electrode fouling is also a major problem in the analysis of biological samples. High-molecular-weight species such as proteins are a major source of fouling, which results in decreasing the sensors response over time. The combination of electrode fouling and interferences presents a formidable challenge to the successful use of electrochemical immuno- sensors.
One-Step FABP Immunotest

Point-of-care (POC) testing is required to have a diagnostic performance comparable to laboratory based cardiac marker assays providing the opportunity for POC testing to evolve into the standard of care for evaluating large numbers of patients presenting with chest discomfort. Although the immuno-affinity filtration chromatography is performed within minutes, it requires sample pretreatment and washing steps. Therefore, a one-step quantitative FABP immunoassay should be developed for practical use in diagnostic medicine.

A rapid and quantitative immunotest for the detection of H-FABP has been successfully developed in our group, in collaboration with sens.biognostic AG (Berlin, Germany, www.biognostic.com). The immunotest is constructed with four main elements: the sample pad, the conjugate release pad, the analytical membrane, and the absorbent pad.

A specific capture monoclonal antibody for H-FABP is immobilized as a test line on NC membrane. Anti-mouse IgG is immobilized as a control line on the same membrane. Another specific detector monoclonal antibody for H-FABP coupled to colloidal gold is impregnated on conjugate pad. The test-strip is then placed into a cassette and ready for use.

When sample is applied to a sample pad, it rapidly wets through to the conjugate pad and the detector reagent is solubilized. The detector reagent begins to move with the sample flow front up the NC membrane. If H-FABP is present in the sample, it binds to the detector antibody. As the sample passes over the test zone to which the capture antibody is immobilized, the analyte–detector reagent complex is subsequently bound. The excess detector reagent is trapped by the control zone. Two red bands at the test and control zones are developed with no further addition of reagent. If the sample contains H-FABP with concentration below the detection limit, only one band at the control zone is visualized. If there is no band developed at both zones, the test is invalid.

The intensity of the test line is in proportion to the amount of H-FABP present in the sample. The control zone acts as a positive control to assure that functional, conjugated antibody migrated throughout the system. The total assay time is less than 10 min. The estimation of the test-strip results can either be performed visually by the naked eyes or by measuring the absorption of the colored band with a Personal Analyzer for Rapid Tests (PART) which is a portable reflection meter with a selected wavelength developed by LRE Technology Partner GmbH (Munich, Germany). It measures the assay results quantitatively.
by scanning the intensity of the red line(s) inside the rectangular window of the cassette and automatically scans to locate an area that has the highest color density, which corresponds to the presence of labelled reagent, thus the concentration of the analyte in the sample can be measured.

The calibration curve of the immunotest was found to be linear up to 125 μg/L H-FABP with \( r^2 = 0.9832 \). The detection limit of the assay was 2.8 μg/L. It was the H-FABP concentration corresponding to a signal 3 SD above the mean \( (n = 20) \) for a sample in which H-FABP was absent, e.g., zero calibrator. Moreover, two different human serum pools with elevated H-FABP concentration were analyzed to estimate the imprecision of the assay on 20 consecutive days. For the pooled serum sample with a relatively low H-FABP concentration (4 μg/L), the calculated intra-assay coefficient of variation (CV) was 10%, and the inter-assay CV was 15%; for the pooled serum sample with a high H-FABP concentration (103 μg/L), the intra-assay CV was 8% and the inter-assay CV was 10%.

Fifty one human samples from patients presenting with chest pain were obtained from the Coronary Care Unit (CCU) of the Prince of Wales Hospital of Hong Kong. The study protocol was approved by the Clinical Research Ethical Committee of the Faculty of Medicine, the Chinese University of Hong Kong. The protocol was thoroughly explained to the patients, and signed consent was obtained. The human samples with H-FABP concentrations between 2 and 285 μg/L, as assessed with the conventional ELISA, were re-evaluated using the newly developed immunotest. As shown in Fig. 5, a good correlation between the two methods was found \( (r^2 = 0.9585) \).

With a performance time of 10 min, the immunotest is much faster than other H-FABP assays published to date. The test can be stored at 4°C and room temperature for up to one year without significant loss of activity. Moreover, no time-consuming sample dilution is necessary, as the assay measures in neat human serum using centrifuge for separation. In order to further simplify the assay procedure, a forthcoming improvement of the assay is aiming at measuring whole blood samples.

A novel one-step FABP test so-called CardioDetect which was derived from the serum immunotest has been successfully developed. It is a rapid chromatographic immunoassay designed for qualitative determination of H-FABP in whole blood samples. After application of three drops of blood onto the test-strip, the result is available within 15 min. The whole blood immunotest requires no sample pretreatment and thus can be applicable to diagnosis of AMI in the emergencies. It may allow
Blood samples obtained from 39 patients admitted to the hospital with chest pain (21 AMI and 17 nonAMI) were studied. Using an upper reference level of 7 mg/L, the specificity of the rapid test was 94%. Both sensitivity and negative predictive value (NPV) were 100% implying that 100% of nonAMI patients can be excluded with no false negative results.

For practical use and self-testing, a credit card design was constructed having two subtests integrated as shown in Fig. 6. The German company rennesens GmbH (Berlin) has acquired a license for the production and distribution of the CardioDetect® from 8sens.biognostic AG. It will be sold in Germany soon.

**CONCLUSION**

Despite the growing amount of information and public awareness on the prevention of certain risk factors for coronary heart disease, it remains the world’s number one cause of death.[1] Faced with a patient complaining of acute chest pain, even today’s cardiologists at times find it difficult to decide whether the pain might be due to an acute myocardial infarction or simply noncardiac pain. This problem becomes even more relevant in the hectic atmosphere of a busy district general hospital.
Therefore, a one-step FABP immunotest has been developed for early
diagnosis of AMI.

Probably the most important trend in diagnostic medicine to emerge
in the last decade has been the shift away from the central hospital
laboratory as the principal test site. An increased proportion of diag-
nostic testing is now being performed in the physicians’ offices or even
at home. Thus, an instant FABP check will have an important role for
AMI diagnosis.

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