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Development of a plasma panel test for detection of human myocardial proteins by capillary immunoassay

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Abstract

A chemiluminescence immunoassay for the detection of four heart marker proteins: myoglobin, creatine kinase mb [CKmb], troponin I [TnI], and fatty acid-binding protein [FABP], was designed. The immunoassay was based on enzyme-linked immunosorbent assay [ELISA] and antibodies immobilized in glass capillaries pre-treated with 3-aminopropyltriethoxysilane. The protein bound to the antibody was detected by using an anti-protein-horseradish peroxidase [HRP] conjugate. The reaction of the HRP with luminal and hydrogen peroxide-based substrate generated the chemiluminescence and a photodiode detector was used to measure the light intensity. The same assay protocol was used to detect all four proteins. Ultrasound waves were used to improve the silanization of glass and the antibody immobilization process. The optimization of the duration and intensity of the ultrasound was performed for the myoglobin assay. Ultrasound improved the silanization procedure and the capillaries gave an approximately 2.5 times greater ELISA response. Ultrasound also improved the sensitivity by approximately 100% when monoclonal antibody was immobilized on a glass capillary. Calibration curves corresponding to analyte concentrations ranging from 2.4 to 2400 ng/ml in plasma samples were recorded. The detection limits were in the region of 1.2 myoglobin, 0.6 CKmb, 5.6 TnI, and 4 ng/ml FABP in plasma with a coefficient of variation of 3–9.9%.

Keywords: Myoglobin; Creatine kinase mb; Troponin I; Fatty acid-binding protein; Chemiluminescent ELISA; Ultrasound

1. Introduction

Patients with typical angina and chest pain accompanied by a marked ST segment (part of an electrocardiogram) are cases who, to a highly probability, should be diagnosed as acute myocardial infarction. In up to 50% of these patients, the electrocardiogram is non-diagnostic and about 4% of patients with developing myocardial infarction are inappropriately sent home (Lee et al., 1987). The diagnosis is based on biochemical markers (Gibler et al., 1992). However, the traditional biochemical markers such as CKmb and myoglobin suffer from low cardiac specificity and sensitivity (Bakker et al., 1993).

The cardiac troponins are now universally accepted as important determinants for the diagnosis of these patients (Alpert et al., 2000). Test systems for TnI promise higher diagnostic efficacy and have certain advantages: a wider diagnostic window of time with early appearance and prolonged appearance, no cross-reactivity of skeletal isoforms and the lack of TnI in skeletal muscle tissue (Heeschen et al., 1998). However, the early diagnosis of acute myocardial infarction [AMI], especially within 3–6 h after its onset, is still problematic in those patients without an obvious elevation of the ST segment. For example, troponins and CKmb have been reported not always to be sensitive for cardiac damage shortly after the onset of AMI (Fransen et al., 1999), and myoglobin detected shortly after the onset of AMI has been reported to be less specific for myocardial damage (Van Nieuwenhoven et al., 1995).

Heart-type fatty acid-binding protein (FABP) is an early cardiac marker of AMI (Adams et al., 1993). The level of FABP is significantly elevated above its threshold level within approximately 3 h after the first clinical symptoms of AMI, and generally returns to a normal value within 12–24 h (Kleine et al., 1992).

An enzyme-linked immunosorbent assay (ELISA) test system for the detection of four cardiac proteins, TnI, myoglobin,

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CKmb and FABP, provides high cardiac specificity and analytical sensitivity for the detection of myocardial injury on a time scale of 3–72 h after onset of chest pain.

An important factor for a conclusive diagnosis has been the specificity of the analytical technique conferred by the antigen-specific antibodies. Immunoassay is typically carried out on microtiter plates. To increase throughput and reduce consumption of limited samples, an effort has been made to adapt ELISA to the microchip format using micro-fluidic systems, and chips bearing micro-wells (Mobini Far et al., 2005). In the recent past, the silanized glass method has offered interesting possibilities for the immobilization of bio-molecules. The ability of silica-based matrix to excellent binding to bio-molecules, while providing a porous skeleton for anchoring the molecules, has been established in several reports (Momeni et al., 1999).

Sensitivity and reproducibility are the most important indicators of the quality by the far greater number of assays. Application of ultrasound to biotechnology is relatively new, but several processes that take place in the presence of cells or enzymes are activated by ultrasonic waves. High intensity ultrasonic waves break the cells and denature the enzymes. Low intensity ultrasonic waves can modify cellular metabolism or improve the mass transfer of reagents and products through the boundary layer or through the cellular wall and membrane (Sinisterra, 1992). The rate-limiting step in many solid-phase immunoassays is associated with the slow kinetics of binding of micromoles, antigen and conjugate to the immobilized phase. The use of ultrasonic energy to enhance mass transport across liquid/solid interfaces can dramatically accelerate antigen binding to immobilized antibodies (Chen et al., 1984). The erosive action of cavitational ultrasound alters the surface properties of the carrier material (Schmidt et al., 1983).

In this report, we demonstrate the usefulness of an ultrasound, capillary-based immunoassay for the measurement of the four cardiac proteins. The light of chemiluminescence is realized by the reaction of hydrogen peroxide and luminol in the presence of horseradish peroxidase [HRP]. The immobilization of antibodies on the glass capillaries is achieved by a silane or glutaraldehyde layer. The glass capillaries were placed vertically with one end facing a photodiode. This arrangement provides appropriate sensitivity due to the light guide properties of the silane-coated capillaries. Ultrasound provides a useful tool to improve the sensitivity and reproducibility of the assay in capillary immunoassay.

2. Experimental details

2.1. Chemicals

Human creatine kinase mb isoform [CKmb] was purchased from Scipac, UK. Anti-CKmb-specific Mab CK 36 and anti-CK-BB-specific Mab, CK33–horseradish peroxidase conjugate were procured from LabAs. Fatty acid-binding protein, monoclonal mouse anti-human FABP, monoclonal mouse anti-human FABP–HRP conjugate, human myoglobin, monoclonal mouse anti-human cardiac myoglobin and monoclonal mouse anti-human cardiac myoglobin-HRP conjugate, troponin I [TnI] antigen (TIC complex), anti-TnI cardiac mAb and monoclonal mouse anti-cardiac TnI–HRP conjugate were purchased from Hytest, Finland. Plasma was obtained from the University Hospital, Lund, Sweden.

Casein blocking buffer and super signal ELISA Femto chemiluminescent substrate were procured from Pierce, Rockford, IL, USA. Procline 300 was from Supelco, PA, USA. Sodium borate buffer in tablet form and washing buffer diluted from tablets were bought from Medicago AB, Sweden. Na_2HPO_4/NaH_2PO_4 were obtained from Merk. BSA came from Sigma, Sweden. Glutaraldehyde and 3-aminopropyltriethoxysilane A-3648 98% were purchased from Sigma. All other chemicals were of analytical grade and were bought from Sigma.

2.2. Materials and apparatus

The glass capillaries, 7.5 cm long, thin walled, 0.9 mm i.d., came from World Precision Instruments, Inc., USA, and the sealing wax was from Sigma. The photodiode chip was supplied by Hamamatsu, the detector and capillary holder were designed by Prolight Diagnostics AB, and produced at a local workshop, Lund, Sweden. The ultrasonic bath was produced by Branson Ultrasonic Corporation, USA, model 3510E-MTH, frequency 40 kHz. The 384-well microtiter plates, black Maxisorp were obtained from Nunc, Denmark. The Victor^{2TM} plate reader was produced by Wallace, Turku, Finland.

2.3. Methods

2.3.1. Coating 3-aminopropyltrimethoxysilane on glass capillaries

Fifty capillaries were placed in a closed glass tube and filled with 40 ml of 6.5% nitric acid. The tube was immediately hanged in an ultrasonic water bath at 70 °C for 10 min, and then washed with 40 ml ultra-pure, distilled water four times. Each time the tube was filled with distilled water and shaken in a Lab quake shaker for 5 min at 500 rpm. The capillaries were then dried with tissue paper and placed in a Hotbox oven for 2 h at 100 °C.

The coating of the glass capillaries with silane was carried out using two different approaches, i.e. by applying ultrasound/heat and only heat. Silanization is performed in a closed glass tube filled with 40 ml solution of 10% 3-aminopropyltriethoxysilane in dried toluene for 30 min at 80 °C in a water bath with or without the application of ultrasound. Capillaries were then washed with 40 ml toluene, followed by 40 ml ethanol 95% and three times with 40 ml ultra-pure, distilled water for each washing step, while the rest of solution, held by capillary force, was adsorbed with tissue paper. The capillaries were then dried in an oven at 100 °C for 2 h. They could be stored in a closed test tube at 4 °C for a long time.

2.3.2. Coating of silanized glass capillaries with glutaraldehyde

The capillaries were filled with a solution of 2.5% glutaraldehyde in Na_2HPO_4, 0.1 M, at pH 7.0. It was allowed to react with the silanized surface at 22 °C for 30 min, and was then washed