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Immunochromatographic Rapid Analysis of Human Heart-Type Fatty Acid-Binding Protein for Acute Myocardial Infarction Diagnosis

E. A. Yakovleva, I. P. Andreeva, V. G. Grigorenko, and A. P. Osipov

Division of Chemical Enzymology, Department of Chemistry, Moscow State University, Moscow, Russia

e-mail: yakovlevahelena@gmail.ru

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Abstract—A method of immunochromatographic assay for rapid detection of human heart-type fatty acid-binding protein (h-FABP) as an early marker of acute myocardial infarction was developed. Gold nanoparticles were used as a visual label. The optimum conditions for assay were determined. The limit of detection in the assay was 1.5 ng/mL, and the variation coefficient did not exceed 8%. Using the developed test system, the clinical diagnosis of acute myocardial infarction ($N = 10$) was confirmed, and the results of testing the serum of healthy individuals ($N = 25$) were negative.

Keywords: immunochromatographic assay, acute myocardial infarction, human fatty acid-binding protein.

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Early diagnosis of acute myocardial infarction (AMI) is one of the important tasks of modern cardiology. The numerous studies established that early treatment of the patients with confirmed AMI can reduce the mortality both in the first hours, and in the later stages of the patient staying in a hospital.

The AMI progression is accompanied by an extensive destruction of cardiomyocytes, and, as a consequence, a high release of the myocardium-specific proteins into the blood. Human heart-type fatty acid-binding protein (h-FABP, 15 kDa) belongs to the family of intracellular lipid-binding proteins involved in the fatty acid metabolism and is abundant in cardiac muscle (0.56 mg per gram of heart tissue). The level of h-FABP in the blood of healthy people does not exceed 6–8 ng/mL [1, 2]. It was shown that upon myocardium injury the h-FABP concentration in the blood like that of myoglobin increases significantly (ten and more times) within 3 h after the onset of AMI symptoms and returns to normal within 12–24 h [3]. This fact, as well as a good tissue specificity compared to myoglobin, makes h-FABP to be a promising marker for early AMI diagnosis.

At present, there are both the quantitative [4, 5] and qualitative methods [6, 7] for determination of h-FABP concentration in plasma (serum) of blood or urine, which are based on the immunochemical assay. Previously, a competitive enzyme-linked immunosorbent assay (ELISA) for determination of h-FABP content in serum was developed in our laboratory [8]; the limit of detection was 1.5 ng/mL. A limitation of this method is a prolonged analysis time, which makes it impossible to perform the rapid determination of h-FABP in the first hours of disease.

In recent years, for the rapid detection of biologically active compounds and diagnosis of many diseases, a visual immunochromatographic assay (ICA) has been widely used in clinical practice. This method provides a short analysis time (10–15 min), simple and accessible detection procedure, and does not require expensive equipment and highly skilled personnel.

The present work is concerned with design of an immunochromatographic test system for fatty acid-binding protein aimed at the early AMI detection.

EXPERIMENTAL

We used chloroauric acid (CAA, Fluka, Switzerland); bovine serum albumin (BSA), sodium citrate, pH test strips, biochemical and chemical reagents (Sigma, United States); and inorganic salts of high-purity grade (Khimmed, Russia). Two types of monoclonal antibodies against h-FABP (MAb⟨9F3⟩ and MAb⟨10E1⟩), two types of polyclonal antibodies against h-FABP (PAb⟨K5⟩ and PAb⟨K10⟩), and anti-species sheep antibodies against mouse IgG were provided by ZAO NVO Immunotekh (Russia). Membranes (MDI, India) were used in the multi-membrane test strips for immunochromatography.

The following buffer solutions were used: 0.01 M potassium phosphate, pH 7.0 (PB); 0.01 potassium phosphate, 0.15 M NaCl, pH 7.2–7.4 (PBS); and 0.01 M potassium phosphate, 0.15 M NaCl, 0.1% Tween-20, pH 7.4 (PBST). The standard h-FABP

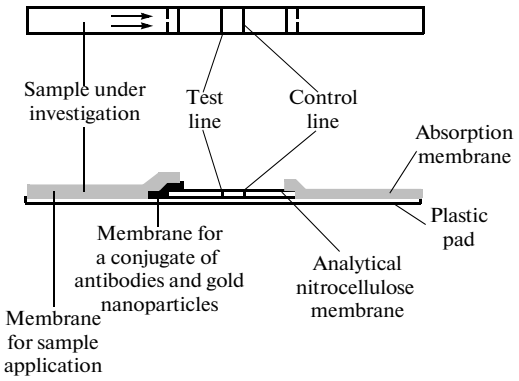

Fig. 1. Schematic representation of the test strip.

solutions of the following concentrations: 0, 2.5, 5, 10, 20, 50, and 100 ng/mL were prepared in PBST or blood serum without h-FABP from the initial h-FABP solution (10000 ng/mL). Serum samples were provided by the Moscow hospitals nos. 50 and 67.

Preparation of colloidal gold. Solutions of colloidal gold with a given particle size were obtained according to the Frens method [9]. Bi-distilled water (99 mL) and 1% CAA (1 mL) were placed in a flask with a magnetic stirrer. The solution was heated to boiling, and then 1% aqueous sodium citrate (2 mL) was quickly added under stirring. The solution was boiled 15 min more under stirring, and then cooled to room temperature in the dark. The average size of obtained nanoparticles was 20 nm according to the scanning electron microscopy.

Determination of the optimum conditions for sorption of antibodies on gold nanoparticles. Optimization was carried out in a 96-well polystyrene plate (Greiner, Austria). In a plate, 8 rows of 6 wells each were selected. Each row corresponded to a fixed pH value; and each column, to a specific concentration of antibodies.

In 2.5-mL aliquots of the colloidal gold suspension, the pH values were varied in an increment of 0.5 in the range from 5.5 to 9.0 by adding 0.1 M solution of sodium carbonate. The solutions of colloidal gold with corresponding pH (200 μL of each) and the antibodies solutions with concentrations of 0, 30, 60, 120, 180, and 360 μg/mL in the deionized water (20 μL of each) were placed in the wells. A plate was incubated for 15 min under stirring, and then 50 μL of 10% NaCl was added to each well. The optical absorbance in each well was measured at wavelengths of 520 and 580 nm.

Preparation of a conjugate of gold nanoparticles and antibodies. A solution of antibodies with a selected concentration (1 mL) was added dropwise to 10 mL of a solution of colloidal gold with pH 7.0–7.5 under stirring and stirred for 30 min at room temperature. Then, BSA (to a final concentration of 0.1%), sucrose (to a final concentration of 10%), and 0.01% sodium azide were added to the resulting solution. The conjugate obtained was stored at +4°C.

To separate the unbound antibodies, the conjugate was centrifuged (20 min, 11000 g, 4°C). The supernatant was removed; the precipitate was re-dissolved in required volume of PB containing 0.1% BSA, 10% sucrose, and 0.01% sodium azide. The absorbance spectrum was measured, and 10 μL of the obtained solution with $A_{520} = 2$ opt units was applied to a fiberglass membrane strip of size 4 × 4 mm.

Preparation of immunochromatographic composite. In order to form the analytical area, a solution of specific antibodies in PBS was applied to the analytical nitrocellulose membrane using a BioDot XYZ 3050 programmed automatic dispenser equipped with a BioJet Quanti 3000 pump (BioDot, United States). To form the control area of immunochromatographic system, a solution of the affinity purified anti-species sheep antibodies against mouse with a concentration of 1 mg/mL was applied at a distance of 5 mm from the analytical area. The following parameters of a BioJet Quanti 3000 pump were used: a drop volume of 30 nl, an increment of 0.3 mm, and a speed of 50 mm/s. The strips were dried at 37°C (relative humidity 25–30%) for 24 h and stored at room temperature in the sealed packages.

The solutions of conjugates of antibodies and colloid gold nanoparticles were applied to the RT-5 membranes and dried in air at room temperature. The test strips were assembled according to the scheme shown in Fig. 1.

Performance of immunochromatographic assay. Ready test strips were placed vertically into the wells of ELISA polystyrene plate with 180 μL of the standard h-FABP solutions in PBST or 180 μL of the test serum solutions preliminary diluted 5-fold with PBST. Within 15 min, the test strips were placed on a horizontal surface and dried.

For quantitative estimation of the assay results, the test strips were scanned on an Epson Perfection V700 Photo flatbed scanner (Seiko-Epson, Japan); images obtained as the graphic files (tif) were processed using

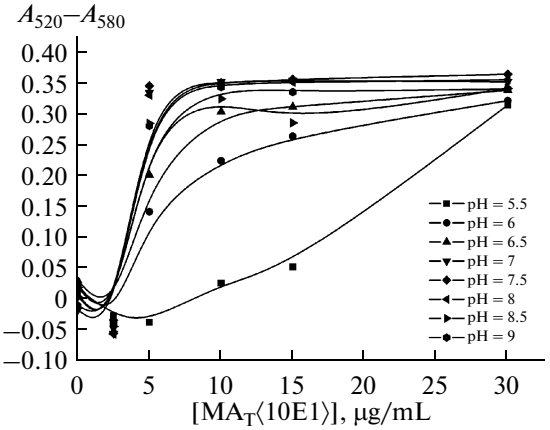

Fig. 2. Relationships between the difference in optical absorbance ($A_{520}-A_{580}$) and the MAb⟨10E1⟩ concentration for different pH values.

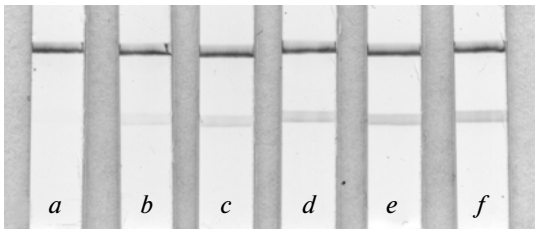

Fig. 3. Results of the determination of the standard h-FABP solutions, ng/mL: *a*, 0; *b*, 5; *c*, 10; *d*, 20; *e*, 50; and *f*, 100.

Scion Image software; the color intensity of the lines in analytical area was measured; and the calibration curves of the relationship between the intensities of the lines and the concentrations of the standard h-FABP solutions were built.

The limit of detection (ng/mL) was calculated as an h-FABP concentration corresponding to two arbitrary units of the signal intensity.

RESULTS AND DISCUSSION

Immunochromatographic assay is based on the interaction of the determined antigen (h-FABP) with antibodies; gold nanoparticles used as a visual detecting agent have the property to absorb light in the visible region of the spectrum. The test strip for the ICA is a pad with the membranes stuck consistently (Fig. 1), along which a flow of liquid containing the test sample moves.

When the sample is applied to a special membrane, its movement along a strip starts by capillary action. In the presence of the determined antigen in the sample, a complex of antigen and gold nanoparticles labeled with specific antigens absorbed on a fiberglass membrane is formed. Then, binding of the formed immunocomplex to specific antibodies immobilized as a narrow line occurs, and a colored line appears, its intensity is proportional to the amount of analyte in the sample. An excess of unbound labeled antibodies migrates further to the control area, where anti-species antibodies are immobilized, and the second colored line is formed. In the absence of h-FABP in the sample, the gold-labeled antibodies are kept only by secondary antibodies in the control area. Thus, the presence of visible color in the control area in both cases points to the operability of the test.

In the present work we used two types of polyclonal antibodies (PAb⟨K5⟩ and PAb⟨K10⟩) and two types of monoclonal antibodies (MAb⟨9F3⟩ and MAb⟨10E1⟩) against h-FABP. To prepare the stable gold-labeled antibodies able to interact efficiently with h-FABP, the optimum conditions (minimum stabilizing concentration and sorption pH) should be selected first. The sets of colloidal gold solutions with different pH (from 5.5 to 9.0) were prepared and specific antibodies were added in different concentrations (from 0 to 30 μg/mL). With increasing ionic strength by adding sodium chloride, the aggregation of unstabilized gold nanoparticles occurred, which was accompanied by a change of the solution color from red to gray-blue, up to colorless. For stabilized gold nanoparticles, the characteristic peak of plasmon resonance is in the region of 520 nm. Therefore, to determine the quantitative stabilization effect, difference of optical absorbance of a solution at wavelengths of 520 and 580 nm ($A_{520}-A_{580}$) was calculated.

Figure 2 shows the data obtained for MAb⟨10E1⟩ monoclonal antibodies against h-FABP. The similar results were obtained for PAb⟨K5⟩, PAb⟨K10⟩, and MAb⟨9F3⟩ antibodies. It should be noted that with increasing concentrations of antibodies, the effect of pH from 7.5 on the stabilization reduced. For preparation of the labeled antibodies, we chose pH 7.0–7.5 for all types of antibodies.

In the studied range of the sol loading by antibodies within 0–30 μg/mL, the minimal saturating concentration of antibodies, that provides the highest sol stability to the coagulating action of electrolyte, is determined by a curve reaching its plateau. It was 10 and 15 μg per milliliter of the sol for MAb⟨10E1⟩ and MAb⟨9F3⟩, respectively; and for PAb⟨K5⟩ and PAb⟨K10⟩ polyclonal antibodies it was 10 μg/mL. These concentrations were used in preparing the conjugates of antibodies and gold nanoparticles.

At the next stage, we selected a pair of antibodies immobilized on the phase and labeled with gold nanoparticles ensuring the maximum sensitivity of the assay of h-FABP. We studied all possible combinations, when one of the antibodies was applied to the phase and a conjugate was prepared with other antibodies. ICA of the standard solutions of h-FABP in PBST was carried out for each pair of antibodies (Fig. 3). The best limit of detection and the highest signal were obtained for the pair of monoclonal antibodies,

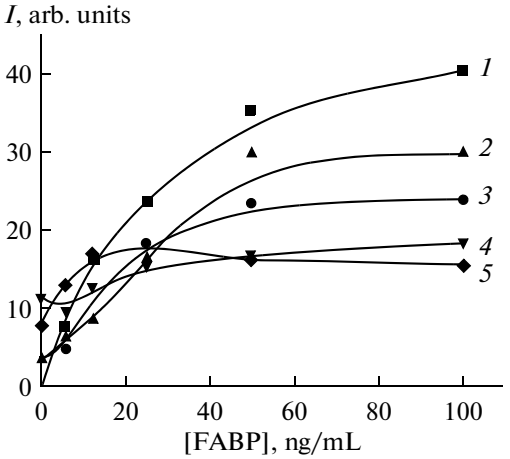

Fig. 4. Calibration curves for the h-FABP determination: *1*, immobilized MAb⟨10E1⟩, conjugate with MAb⟨9F3⟩; *2*, PAb⟨K5⟩, conjugate with MAb⟨9F3⟩; 3, MAb⟨9F3⟩, conjugate with MAb⟨10E1⟩; 4, PAb⟨K5⟩, conjugate with MAb⟨10E1⟩; and 5, PAb⟨K5⟩, conjugate with PAb⟨K10⟩.

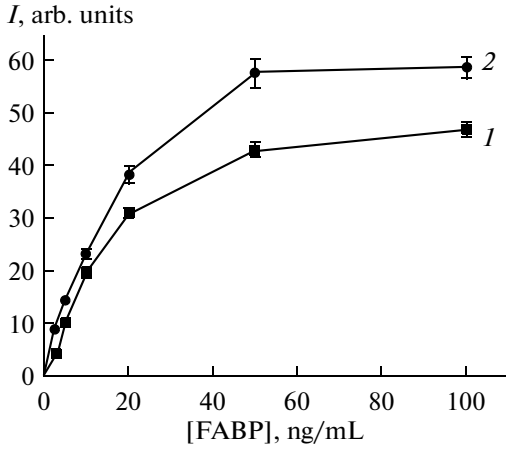

Fig. 5. Calibration curves for the standard h-FABP solutions prepared in: *1*, the serum without h-FABP with 5-fold dilution; *2*, PBST.

MAb⟨10E1⟩ immobilized on a membrane and MAb⟨9F3⟩ conjugated with gold nanoparticles (Fig. 4, curve *1*). In case of PAb⟨K5⟩ polyclonal antibodies immobilized on a membrane in a combination of the same conjugate, the signal reduced markedly and a background signal was observed (Fig. 4, curve *2*). The assay results for labeled MAb⟨10E1⟩ were worse with both monoclonal and polyclonal antibodies (Fig. 4, curves *3* and *4*, respectively). When we used the polyclonal antibodies immobilized on a membrane and conjugated with gold nanoparticles, for example PAb⟨K5⟩ with Au–PAb⟨K10⟩ conjugate (Fig. 5, curve *5*), we observed a low main signal, a background signal, and the lowest sensitivity of analysis.

In our further work we selected two membranes (analytical and for sample application) and optimized the concentrations of specific immunoreagents and the conditions for antibodies immobilization on a membrane. As the concentration of absorbed MAb⟨10E1⟩ (from 0.25 to 1.0 mg/mL in PBS) and labeled MAb⟨9F3⟩ (from 5 to 10 μL per a strip) antibodies increases, so do the intensity of detected signal and the slope of the calibration curve at the initial segment, which is typical for the sandwich-type assays. However, at the MAb⟨10E1⟩ concentration of 1 mg/mL, we observed a background signal, i.e., the presence of nonspecific interaction in the absence of h-FABP in the sample solution, which is essential for visual evaluation of the assay results. So, the following conditions were chosen as the optimum: MAb⟨10E1⟩ in a concentration of 0.5 mg/mL for immobilization on a membrane, and gold-labeled MAb⟨9F3⟩ in

Results of the immunochromatographic assay of h-FABP in the serum of AMI patients

Experiment number	h-FABP				
	on admission	within 6 h	within 12 h	within 18 h	within 24 h
50-010	+	+	+	–	–
50-028	+	+	+	–	–
50-039	+	+	+	+	–
67-004	+	+	+	+	–
67-006	+	+	–	–	–
67-007	+	+	+	–	–
67-009	+	+	+	+	+
67-010	+	+	–	–	–
67-013	+	+	+	+	–
67-014	+	+	+	+	–

Note: (+)—positive, (–)—negative.

amounts of 7.5 μL (A_{520} = 2 opt units). Fig. 5 (curve *1*) shows a representative calibration curve. The developed method makes it possible to determine the h-FABP concentration in the range of 2–100 ng/mL. The limit of detection for h-FABP was 2 ng/mL. The method is characterized by good reproducibility, and the coefficient of variation for standard h-FABP concentrations did not exceed 8%.

To validate the developed ICA, we conducted a determination of h-FABP in the blood serum of healthy individuals (control group) and patients with clinically confirmed AMI. First, we studied the effect of serum components on the analytical results and showed that the sensitivity of assay and the analytical signal increase compared to the buffer solution. In this case, the limit of detection was 1.5 ng/mL, which corresponds to 7.5 ng/mL of h-FABP in the sample of blood serum.

When analyzing the blood serum in the control group, we observed only one control line in the analytical area of test strips. This indicates that the concentration of h-FABP in these samples was below the limit of detection in our method (7.5 ng/mL). When analyzing the samples of blood serum of the patients with confirmed AMI, the test showed positive results (two colored lines in the analytical area) for all samples (see the table). Blood serum samples were taken on admission of patients to the hospital and then every 6 h. A decrease of the signal intensity in the test zone was observed within 6 h, and the excess of the threshold concentration of h-FABP was within 24 h in only one patient.

Thus, the method developed for the rapid determination of h-FABP can be successfully applied in the clinical diagnostic laboratories of cardiology hospitals as well as at the emergency and cardiac intensive care units to confirm or reject the diagnosis at the early stages of AMI (from the first hour and later).

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