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(57) Abstract

The present invention provides a method for detecting myocardial necrosis by incubating a sample with a natural binding partner (troponin C) for troponin I and antibodies to troponin C and troponin I; and determining the resultant immune complex formed in proportion to the total concentration of troponin I in the sample.

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METHOD OF DETECTING AND DETERMINING THE CONCENTRATION OF TOTAL TROPONIN I IN A BIOLOGICAL SAMPLE

FIELD OF THE INVENTION

This invention relates to an immunoassay for human cardiac troponin I, wherein the total cardiac troponin I is detected and measured, said assay being used to aid in the diagnosis of acute myocardial infarction, perioperative myocardial infarction, unstable angina (microinfarcts) or other conditions which could lead to injury or infarction of the heart muscle.

BACKGROUND OF THE INVENTION

During the course of a heart attack or, more technically, an acute myocardial infarction (AMI), it has been observed that the human heart muscle releases a protein substance into the bloodstream known as cardiac troponin I (cTnI). This substance is particularly specific to the heart muscle and, absence a heart attack, is almost never found in the bloodstream. Thus, as will be appreciated, the detection of cTnI in the bloodstream is a strong indicator that a patient experiencing chest pains and other heart attack symptoms has, indeed, suffered a heart attack and should be treated accordingly.

Structurally different proteins of the cardiac troponin complex, namely, cTnI and troponin T (cTnT) have been evaluated as potential markers for acute myocardial infarction (Mangano, D.T. 1994, Anesthesiology 81, 1317-1320). A third troponin component, troponin C (cTnC), completes the complex. Conformational changes in the troponin complex occur when cTnC binds calcium, thus enabling the contractile interaction between actin and myosin.

Recently, it was reported that much of the cardiac troponin I released into the bloodstream during a heart attack is complexed with cardiac troponin C (Katrukha, A. *et al.*, "Cardiac Troponin I - Cardiac Troponin C Complex In Serum Of Patients With Acute Myocardial Infarction", Proceedings of the XVI International Congress of Clinical Chemistry, No. B86, [July 1996]). Unfortunately, the presence and prevalence of cTnC in the cTnI:cTnC complex may make detection of cTnI very difficult since the cTnC can mask epitopes to which a cTnI-specific antibody would otherwise bind.

In order to accurately measure cardiac troponin I in clinical specimens, the specific antibodies must bind both complexed and uncomplexed cTnI with high affinity. Thus an assay which determines the concentration of total cardiac troponin I, that is, complexed and uncomplexed cTnI in a biological sample is required.

UK Patent Application GB2275774A to Pasteur Sanofi Diagnostics, teaches the use of cTnC (bovine) to stabilize cTnI or cTnT in calibrators and controls.

Bodor et al. (Clin. Chem. 38(11):2203-2214, 1992) reported the isolation of monoclonal antibodies which bound cTnI in the presence of rabbit skeletal TnC (sTnC).

PCT/US 94/05468 patent application to Fortron Bioscience Inc. describes a variety of assay formats using TnC as one binding partner and an antibody as the other.

Katrukha, A. *et al., supra*, demonstrated the presence of complexed troponin I in the serum of AMI patients with the use of anti-human cardiac troponin C and anti-human cardiac troponin I antibodies. They also determined the respective concentrations of uncomplexed cTnI and total cTnI using two different combinations of antibodies to cTnI, and a reagent to dissociate the complex.

SUMMARY OF THE INVENTION

In view of the foregoing discussions, an object of the present invention is to provide assay methods and test kits, which are especially useful for detecting and determining the concentration of total cTnI, that is, complexed and uncomplexed cTnI, in a biological sample. One advantage of the present invention is a means to detect and measure the total cTnI in a sample using only one specific anti-cTnI antibody.

The above object is achieved by an assay for detecting and measuring the total concentration of cTnI in a biological sample, which assay comprises an anti-cTnC antibody reagent and an anti-cTnI antibody reagent that binds both complexed and uncomplexed cTnI in the same sample, calcium and/or magnesium, and TnC. Thus, one antibody reagent binds the TnC component, the TnC:cTnI complex from the sample, and the TnC:cTnI

complex formed *in situ* by uncomplexed cTnI and reagent TnC; while the other antibody reagent binds both complexed or uncomplexed cTnI.

In one embodiment of the invention, there is provided an immunometric assay for cTnI in a sample which comprises: including TnC in an assay also containing antibody binding reagents in order to form cTnI:TnC and immune complexes; incubating; separating the bound from unbound reagents; and detecting the bound labeled antibody reagent in proportion to the amount of cTnI in the sample.

The present invention further provides test kits for performing diagnostic assays which are especially useful for detecting and determining the amount of cTnI in a sample, which kits are comprised of troponin C; calibrators and controls; anti-TnC and anti-cTnI antibody binding reagents; divalent cations, preferably calcium together with or separate from magnesium substrate buffers, if necessary; and other reagents required for the desired cTnI assay.

Other objects of the present invention will in part be obvious and will in part appear hereinafter.

BRIEF DESCRIPTION OF THE DRAWINGS

FIGURE 1A shows anti-troponin C bound to the reagent TnC component of the troponin C:cardiac troponin I complex, with the antibody to cardiac troponin I bound to the cTnI component which was contained in a sample.

FIGURE 1B shows the reagent antibody to TnC and reagent antibody to cardiac troponin I bound to their respective components of a cTnC:cTnI complex contained in a sample.

DETAILED DESCRIPTION OF THE INVENTION

While the present invention is satisfied by embodiments in many different forms, herein will be described in detail a particular embodiment of the invention, with the understanding that the following description is to be considered as exemplary of the principles of the invention and is not intended to limit the scope of the invention as determined by the appended claims and their equivalent.

The term "assay" as used herein is defined to include conventional immunological techniques, for example, radioimmunoassay, immuno-radiometric assay, enzyme-labeled immunosorbent assay (ELISA), enzyme-labeled immunometric assay, fluorescence-labeled immunoassay, and luminescence-labeled immunoassay.

The term "antibody" as used herein is defined to include polyclonal antibody from any native source, and native or recombinant monoclonal antibodies of classes IgG, IgM, IgA, IgD, and IgE, hybrid derivatives, and fragments of antibodies including Fab, Fab', and $F(Ab')_2$.

The term "troponin C" as used herein is defined to include troponin C from any native or recombinant source, but is preferably recombinant human cardiac troponin C (rHcTnC).

The term "troponin" as used herein is defined to include troponin obtained from recombinant and native sources. Native troponin includes troponin from any source found in nature.

In carrying out the present invention, the method in more detail comprises: (a) including TnC or another substance that binds to cardiac troponin I, in an assay mixture which contains antibody binding reagents where one antibody binds complexed and uncomplexed TnC and the other binds complexed and uncomplexed cTnI, and a biological sample; (b) incubating the assay mixture for a time and under conditions sufficient to form cTnI:TnC complexes and for the respective antibody reagents to bind such complexes; (c) separating unbound TnC and unbound labeled-antibody reagents from the resultant immune complex with a series of washes; and (d) detecting the amount of labeled antibody reagent bound in the immune complex in proportion to the concentration of cTnI in the sample by means consistent with the type of label.

Typically, the biological sample may be blood, or blood fractions such as plasma or serum. The assays of the present invention can use a reporter reagent to detect the presence or determine the concentration of an analyte in a test sample and use a capture reagent, directly or indirectly attached to a solid phase material, to separate the binding reaction complex from the test sample and assay reagents for ease of observation.

The antibody capture binding reagent can be in any configuration that enables one to perform direct or indirect capture of cTnI and its complex; e.g., the capture antibody adsorbed or coupled to a plastic microtiter well, antibody adsorbed or covalently coupled to submicron to micron-size paramagnetic or nonparamagnetic particles, or antibody modified with a ligands like biotin or fluorescein for indirect capture using streptavidin- or antifluorescein-coated particles or plastic surfaces, respectively.

The antibody reporter reagent is comprised of the antibody alone, subsequently detected with a secondary binder-label conjugate; an antibody-label, where fluorophores, enzymes, luminigens, and radioisotopes are common examples; or it can be modified with a ligand for indirect detection, for example a biotin label that is subsequently detected with a streptavidin-enzyme conjugate.

Two schematic representations of immune complexes resulting from binding of the respective reagent antibodies to troponin C-cardiac troponin I complexes are shown in Figures 1A and 1B. Figure 1A shows anti-troponin C bound to the reagent TnC component of the complex, with the antibody to cardiac troponin I bound to the cTnI component which was contained in the sample. FIGURE 1B shows the reagent antibody to TnC and reagent antibody of cardiac troponin I bound to their respective complex components of a cTnC:cTnI complex contained in the sample. Both anti-troponin C and anti-cardiac troponin I may serve as part of either the capture reagent or the labeled reporter reagent; in some configurations where there is no capture reagent, both reagent antibodies are labeled.

In a preferred embodiment of the present invention, preferably 100-1000 fmoles of TnC and calcium and/or magnesium (0-10 mM, preferably 1 mM) is included in the component containing one or both of the antibody binding reagents (capture and reporter; usually 1-10 pmoles per assay) for cTnI and TnC, respectively. The binding reagents can be separate or combined.

The assay method can be performed sequentially or simultaneously at 15-40°C. The preferred format is a simultaneous incubation (with agitation) of the TnC, capture and reporter antibody binding reagents with the sample for

5 to 120 minutes. A subsequent incubation with additional capture or reporter reagents could also be performed. After requisite incubations and washes, the label is detected directly, or for example, if an enzyme, by the addition of substrate to the antibody-captured cTnI:TnC immune complex.

The present invention further provides for kits containing the components for performing the assay methods of the present invention. Potentially included in the kits are: calibrators and controls; separate or combined with antibody binding reagents, or antibody solid phase; troponin C, divalent cations, preferably, calcium and/or magnesium; wash buffers; and detection solutions. The detection solutions may contain substrates for enzymes, whereby the products of catalysis elicit a change in signal relative to the substrate.

EXAMPLES

The examples described below demonstrate: 1) that an anti-human cardiac troponin C monoclonal antibody capture phase and an anti-recombinant human cardiac troponin I monoclonal antibody-enzyme conjugate bind the troponin C:I complex as a function of rHcTnC concentration, and 2) at an optimal level of reagent rHcTnC, equimolar concentrations of troponin I as C:I, (C:I + I), and completely uncomplexed troponin I elicit similar responses in a simultaneous immunometric sandwich assay.

EXAMPLE 1. Monoclonal Antibodies.

Monoclonal anti-human cardiac troponin C (IgG2a) Clone 1A2 were purchased from Biodesign International, Kennebunk, ME; and monoclonal anti-human cardiac CTnI antibodies may be purchased from Biodesign International, Kennebunk, ME.

Murine ascites fluid containing monoclonal antibody (Mab) from anti-rHcTnI Clones 5D12-1C2 (designated 1C2) was obtained from the University of Miami Medical Center, Miami, FL. The antibody was purified by Protein A affinity chromatography as follows: The ascites fluid was diluted with an equal volume of Affi-Gel® Protein A MAPS® II Binding Buffer (Bio-Rad Laboratories, Hercules, CA), clarified, and applied to a 10 mL agarose-

immobilized Protein A column (Immunopure® *Plus* , Pierce, Rockford, IL) equilibrated with the Binding Buffer. Under gravity, the column was washed with the same buffer until the absorbence at 280 nm baselined. Monoclonal antibody was eluted from the Protein A column with 50 mM potassium phosphate, pH 7.0, - 120 mM NaCl ("KPBS") and then concentrated on a YM-30 membrane in a concentration cell (Amicon® Inc., Beverly, MA). The antibody was filtered through a 0.2 μ low-protein binding Acrodisc® membrane (Gelman Inc., Ann Arbor, MI) and quantitated at 280 nm where 1.4 A_{280} is 1.0 mg/mL.

EXAMPLE 2. Antibody-enzyme conjugate 1C2-alkaline phosphatase.

Bovine intestine alkaline phosphatase obtained from Calbiochem (La Jolla, CA) was dialyzed against 125 volumes of a buffer containing 50 mM MES, pH 6.0, 150 mM NaCl, 10 mM $MgCl_2$, and 0.2 mM $ZnCl_2$ at 4°C with 3 buffer changes. The enzyme (10.4 mg) was oxidized with 10 mM sodium periodate in 1.72 mL in the dark in ice for 60 minutes. Oxidation was quenched with 100 μL of 50% glycerol for 30 additional minutes and the oxidized enzyme was desalted on a 72 mL (2x23 cm) Bio-Gel® P-6DG column (Bio-Rad) equilibrated with the dialysis buffer.

The oxidized alkaline phosphatase (9.3 mg) was adjusted to pH 5.0 with 6 N HCl and then polymerized (1.04 mg enzyme/mL) with 4.2 mg of polyacrylamide hydrazide (Sigma) overnight at 4°C. The remaining hydrazides were capped with 0.5 mL of 0.5 M glyceraldehyde for 30 minutes at ambient temperature. After adjusting the pH to 7.0 with 5 N NaOH and increasing the NaCl concentration to approximately 0.5 M, the polymerized alkaline phosphatase (pAP) was concentrated to 4.2 mL in an Amicon concentration cell using a YM-30 membrane (Amicon). It was microfuged prior to gel filtration on a 2.6 x 93 cm Bio-Gel A-1.5 column (fine mesh; Bio-Rad) equilibrated with an alkaline phosphatase conjugate buffer ("AP Conjugate Buffer") containing 100 mM Bis-Tris propane, pH 7.0, 150 mM KCl, 10 mM $MgCl_2$, 0.2 mM $ZnCl_2$, and 0.05% NaN_3. The flow rate was 18 mL/hour under ambient conditions. Five (5) mL fractions were collected. The fractions containing the high molecular weight peak were pooled and concentrated on an XM300 membrane (Amicon) to approximately 2 mL. Toward the end of

the concentration step, 375 µL of 5 M NaCl was added to discourage precipitation. The pAP was then dialyzed overnight against KPBS at 4°C.

The polymerized alkaline phosphatase was activated with a 25-fold molar excess of sulfosuccinimidyl 4-[N-maleimidomethyl] cyclohexane-1-carboxylate (sulfo-SMCC; Pierce Chemical Co.) for 1 hour at ambient temperature. It was then desalted on a 72 mL (2x23 cm) Bio-Gel P-6DG column equilibrated with 100 mM sodium phosphate, pH 6.5-5 mM EDTA, concentrated to 3.7 mL on an XM300 membrane and placed in ice until conjugation.

Nine (9) mg of the monoclonal antibody 1C2 was buffer-exchanged into 100 mM triethanolamine, pH 8.3-1.0 mM EDTA using a Centricon C-30 microconcentrator (Amicon). The antibody was iminothiolated with a 25-fold molar excess of 2-iminothiolane (Pierce) in a 1.0 mL reaction at ambient temperature for 1.0 hour. It was then desalted on a (2x23 cm) Bio-Gel P-6DG column equilibrated with 100 mM sodium phosphate, pH 7.4, and concentrated with a YM-30 membrane to 1 mL.

Conjugation was initiated by adding the pAP dropwise to the thiolated antibody with stirring. The reaction (4.8 mL) proceeded for 3 hours at ambient temperature, then 4°C overnight. Unreacted thiols were capped with 50 µL of 1 mM N-ethylmaleimide (Pierce) for one hour at ambient temperature. After dialysis against the AP Conjugate Buffer overnight at 4°C, the conjugate was separated from the unconjugated components by gel filtration on the Bio-Gel A-1.5 column equilibrated with the conjugate buffer. The flow rate was 18 mL/hour at ambient temperature. The pooled fractions were concentrated on an XM300 membrane, supplemented with 5 mg/mL each of protease-free bovine serum albumin (Sigma) and Triton X-100, and 1 µg leupeptin before it was sequentially filtered through 0.8 µ and 0.22 µ Acrodiscs. The conjugate (1.7 mg at 0.449 mg/mL; 1.0 antibody:4.5 AP) was diluted to 100 pmoles per mL in CIB (50 mM Bis-Tris propane, pH 7.0, 150 mM KCl, 1.0 mM $CaCl_2$, 0.25% bovine serum albumin, 0.05% Tween-20®, and 0.1% NaN_3) prior to use.

EXAMPLE 3. Simultaneous microtiter ELISA using different amounts of cTnC.

Recombinant human cardiac troponin I was provided by the University of Miami Medical School. Prior to assay, the cTnI was diluted to 2 pmoles/mL (23,710 MW) in a cTnI incubation buffer (TIB) containing 50 mM Bis-Tris propane, pH 7.0, 0.5 M NaCl, 1.0 mM $CaCl_2$, 0.5% bovine serum albumin, 0.5% Tween-20, and 0.1% NaN_3. Recombinant human cardiac troponin C was also provided by the University of Miami Medical School.

Immulon®4 Dividastrips® (Dynatech Laboratories, Chantilly, VA) were coated with 10 pmoles of the anti-cardiac TnC antibody in 0.1 mL of KPBS per well and adsorbed overnight at 4°C. The wells were washed 3-4 times with 0.25 mL of phosphate-buffered saline-0.5% Tween-20 (PBST) prior to use. In order, 100 µL of the antibody-AP conjugate (100 pmoles/mL CIB) combined with various amounts of cTnC, and 50 µL of cTnI (2 pmoles/mL) in TIB were placed in the appropriate wells and incubated for one hour at ambient temperature. After washing 6 times with PBST, C:I and immune complex formation was monitored with 0.2 mL of 5 mM p-nitrophenolphosphate (PNPP; Pierce) in 0.3 M diethanolamine, pH 9.8 containing 5 mM $MgCl_2$. Readings at 405 nm were taken after 20 minutes at ambient temperature on a microtiter plate reader. Results are shown below in Table 1.

TABLE 1

Experiment	fmoles cTnC	A405 @ 20 minutes
1	100,000	0.3435
	50,000	0.422
	25,000	0.485
	12,500	0.6535
	6,250	0.918
	3,125	1.3255
	1,563	1.725
	781	2.175
	0	0.181
2	1,000	2.611
	500	2.9195
	250	2.7575
	125	2.4915
	62.5	1.748
	31.3	1.06
	15.6	0.6595
	7.8	0.396
	0	0.169

These results show a response that is a function of the cTnC concentration. The optimum level of cTnC in the assay is 250-500 fmoles when the reaction contains 100 fmoles of cTnI.

EXAMPLE 4. Simultaneous microtiter ELISA for C:I complex, a C:I + I mixture, and I.

Complexed TnC:cTnI was prepared by combining of 0.48 μM cTnC with 0.48 μM cTnI and incubating the mixture in TIB for 30 minutes at ambient temperature before further dilution. Reactions containing both C:I and I were made by mixing equal volumes of complex and 0.48 μM uncomplexed cTnI together. All 3 samples were respectively diluted in TIB and ACD (adenine in citrated dextrose) plasma so that the highest cTnI concentration was 2 pmole/mL (2 nM), before serially diluting each 1:1 with TIB. After adding 100 μL of a combined reagent containing both 10 pmoles of 1C2-AP and 250 fmoles cTnC in CIB to wells containing 10 pmoles of adsorbed anti-cTnC MAb 1A2. The reactions were initiated with 50 μL of the respective sample dilution. The incubation and further processing is as described in the previous experiment. The results are tabulated below in Table 2:

TABLE 2

	A405 @ 20'					
fmole	Buffer			Plasma		
cTnI	I:C	I:C + I	I	I:C	I:C + I	I
50	2.07	2.021	2.07	0.417	0.45	0.499
25	1.225	1.128	0.989	0.243	0.267	0.263
12.5	0.63	0.495	0.52	0.204	0.216	0.190
6.25	0.341	0.275	0.295	0.143	0.152	0.157
3.13	0.234	0.206	0.245	0.140	0.155	0.181
1.56	0.196	0.193	0.184	0.141	0.154	0.135
0.78	0.162	0.21	0.134	0.121	0.144	0.148
0	0.114	0.105	0.115	0.107	0.123	0.121

The respective cTnI dose responses for TnC:cTnI, TnC:c + cTnI, and cTnI alone in either buffer or plasma are essentially the same. The signal increases in response to increases in the cTnI concentration indicating that the reagent

cTnC binds uncomplexed cTnI resulting in a complex bound and captured by anti-cTnC and detected with the anti-cTnI alkaline phosphatase conjugate. The signal for the dose responses in plasma is lower compared to that generated in buffer, presumably due to matrix effects of the plasma. One could perhaps minimize this effect by optimizing the cTnC and antibody reagent concentrations while in the presence of plasma, or using a different anti-TnC antibody. The results in Table 2 show that inclusion of troponin C in the mixture enables one to detect and determine the concentration of total sample cardiac troponin I, that is, the combination of both complexed and uncomplexed sample cTnI in the same assay.

All publications cited in this specification are indicative of the level of skill of those in the art to which this application pertains. Each publication is individually incorporated herein by reference in the location where it is cited.

One skilled in the art will appreciate that although specific reagents and conditions are outlined in the above preparations and methods, modifications can be made which are meant to be encompassed by the spirit and scope of the invention. The preparations and methods, therefore, are provided to illustrate the invention.

CLAIMS:

1. A method for the detection of myocardial infarction by determination of the total amount of cardiac troponin I in a biological sample, which method comprises the steps of:

(a) incubating the sample with (i) troponin C or another troponin I binding substance and (ii) at least two antibody reagents, where one antibody reagent binds troponin C and the other specifically binds cardiac troponin I, either or both of the antibody reagents being labeled, so that troponin C complexes with cardiac troponin I and the respective antibody reagents bind to complexed troponin C:cardiac troponin I;

(b) separating the complex from any unbound reagents ; and

(c) determining the amount of labeled antibody bound in the immune complex as a measure of the concentration of cardiac troponin I in the sample.

2. The method of claim 1 wherein the troponin C is selected from any native or recombinant troponin C.

3. The method of claim 1 wherein the biological sample is selected from whole blood and blood fractions.

4. The method of claim 1 wherein the antibody binding reagents include a capture antibody and a labeled reporter antibody reagent, where one antibody reagent binds troponin C, the troponin C:cardiac troponin I complex formed *in situ*, and the endogenous cardiac troponin C:cardiac troponin I complex, and another antibody reagent binds either complexed or uncomplexed cardiac troponin I.

5. A kit useful for the determination of the total amount of troponin I in a sample, which kit comprises:

(a) calibrators and controls;

(b) antibody binding reagents;

(c) troponin C and a divalent cation;

(d) wash buffers; and

(e) detection solutions.

6. The kit of claim 5 wherein the detection solutions contain substrates for enzymes.

7. The kit of claim 5 wherein the divalent cation is calcium together with or separate from magnesium.

8. The kit of claim 5 wherein the calibrators and controls are prepared by adding troponin C and a divalent cation to cardiac troponin I.

— < TnC:cTnI > —

FIG. 1A

— < cTnC:cTnI > —

FIG. 1B