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A comparison of capture antibody fragments in cardiac troponin I immunoassay

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ABSTRACT

Objectives: To compare cardiac troponin I (cTnI) values measured from 32 normal plasma specimens with a two-site cTnI research assay exploiting different molecular forms of a capture antibody.

Design and methods: The current research assay consists of two capture antibodies immobilized on streptavidin-well surface and one detection antibody attached to highly fluorescent europium(III)-chelate-doped nanoparticles. Four different molecular forms of one of the capture antibodies (intact monoclonal (Mab), F(ab′)$_2$ fragment, Fab fragment and chimeric Fab fragment (cFab)) were tested. The developed immunoassays were evaluated in terms of their analytical sensitivities and assay kinetics. Furthermore, cTnI concentrations were measured from 32 heparin plasma samples from apparently healthy donors (mean age 32; range 24–60 years).

Results: The differences in the measured cTnI concentrations (corrected for the buffer-based zero calibrator) between the Mab and the three fragmented forms were highly significant ($P<0.0001$). Replacing the intact Mab with the antibody fragments also reduced the required antibody amount from 100 ng to 66 ng (F(ab′)$_2$) and 16.5 ng (Fab and cFab). Furthermore, the limit of detection was improved when Fab fragments were employed (Mab: 0.90 ng/L, Fab: 0.69 ng/L and cFab: 0.41 ng/L). The apparent normal range median (minimum/maximum) of the 32 healthy subjects was reduced from 7.28 ng/L (2.64/116 ng/L) with Mab to 1.80 ng/L (0.746/10.6 ng/L) for the cFab.

Conclusions: Eliminating the Fc-part from one of the two capture antibodies in an immunofluorometric cTnI assay substantially reduced the measured cTnI concentrations, simultaneously improving the assay sensitivity and reducing the reagent consumption.

Introduction

Cardiac troponins (cTn) I and T are sensitive and specific biochemical markers for myocardial damage and the markers of choice for triage of acute coronary syndromes and myocardial infarction. However, repeatedly elevated cTn levels that do not correlate with a clinical picture of a cTn-elevating condition, may indicate a false-positive result caused by analytical interference.

The most frequently described analytical interference causing either false-positive or -negative test results in immunoassays includes human anti-animal antibodies, rheumatoid factor (RF) and complement [1,2]. The most common anti-animal antibody in the sera of some patients is human anti-mouse antibody (HAMA). Estimations of its prevalence vary widely (<1–80%) [1]. HAMA is especially problematic in two-site immunoassay using antibodies from murine origin. In that case HAMA can bridge between capture and detection antibodies in the absence of target analyte and cause a spuriously elevated value. Heterophilic antibodies have been reported to display variable specificity towards different IgG subclasses and especially subclass IgG1 seems to be prone to HAMA interference [3]. Positive interference caused by HAMA has been described frequently in cTnI assays [4–6].

Recent assay technologies have enabled measurement of minute concentrations of circulating cTnI in apparently healthy individuals with a 99th percentile limit at 10 ng/L [7,8]. Reliable detection of persistently elevated or dynamic changes at this low concentration range requires effective removal of both positive and negative analytical interferences. The routinely employed way to decrease HAMA interference in commercial immunoassays is to use an excess of scavenger antibody of the same species as used in the assay. Another common approach to avoid HAMA interference in two-site immunoassays is to use F(ab′)$_2$ or Fab fragments as capture and/or detection antibodies instead of intact monoclonal antibodies [9,10]. Lack of the Fc portion eliminates interference from complement and rheumatic factors but only partly from HAMA, which can also be directed against antigenic determinants on the constant parts of the antibody fragments. Also chimeric antibodies with human constant parts replacing the murine ones have been shown to be helpful in eliminating/reducing HAMA interference [11].

The scope of this study was to determine cTnI values from 32 normal plasma samples with a two-site cTnI immunoassay. The previously published research assay [12] consists of two capture antibodies immobilized on streptavidin (SAv) surface and one detection antibody attached to highly fluorescent europium(III)-chelate-doped nanoparticles. This kind

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of an assay concept is potentially prone to nonspecific signal increase caused by matrix related interferences (RF and HAMA). This risk is further potentiated in applications striving towards very low detection limits. Thus, the aim was to investigate the extent of such interferences by exploiting different molecular forms of one of the capture antibodies in the research assay: an intact monoclonal antibody was compared to an enzymatically digested $F(ab')_2$, a recombinant Fab and a mouse/human chimeric Fab (cFab). In addition to cTnI levels measured from normal plasma samples, the developed assays were evaluated in terms of their analytical sensitivities and assay kinetics.

Materials and methods

Materials

Europium(III)-chelate doped Fluoro-Max™ polystyrene nanoparticles (107 nm in diameter), whose fluorescent properties have been described previously [13], were purchased from Seradyn (Indianapolis, IN). N-hydroxysulfosuccinimide (sulfo-NHS) and N-(3-dimethylaminopropyl)-N′-ethylcarbodiimide (EDC) were obtained from Fluka (Buchs, Switzerland). Biotin isothiocyanate was synthesized at the Department of Biotechnology, University of Turku [14]. Bovine serum albumin (BSA) was purchased from Bioreba (Nyon, Switzerland), bovine gamma globulin (BGG; G-5009) and D-sorbitol from Sigma-Aldrich (St. Louis, MO), and native and denatured mouse IgG from Meridian Life Science (Saco, ME). Casein and heparin were purchased from Calbiochem (La Jolla, CA). Low-fluorescent, normal capacity SAv-coated 12-well microtiter strips, and Kaivogen buffer and wash solutions were obtained from Kaivogen (Turku, Finland). All other reagents used were of analytical grade.

Antibodies

The assay utilized three cTnI specific monoclonal antibodies (Mabs) whose epitopes were at amino acid residues 41–49 (clone 19C7; HyTest, Turku, Finland), 137–148 (clone 8I7; International Point of Care, Toronto, Canada) and 190–196 (clone 9707; Medix Biochemica, Kauniainen, Finland). The clones 19C7 and 8I7 were used only as intact Mabs. The clone 9707 was additionally used as $F(ab')_2$ and Fab fragments. The $F(ab')_2$ antibody fragment was produced from the parental 9707-Mab by enzymatic digestion with bromelain (ID-Diluent 1; Diamed, Switzerland) as described by Väisänen et al. [15]. Mabs 19C7 and 9707 were biotinylated with a 10-fold and $F(ab')_2$-fragment of 9707-Mab with a 30-fold molar excess of biotin isothiocyanate using a procedure described earlier [16]. Recombinant Fab fragment of 9707 was cloned from the hybridoma cell line of Medix Biochemica and produced at the Department of Biotechnology, University of Turku (Turku, Finland), as reported previously [10]. Fab-9707 was further modified by replacing constant parts of the light and heavy chains with corresponding human antibody sequence to provide mouse/human antibody chimera (cFab-9707). Both Fab fragments were designed with an unpaired cysteine residue in the C-terminal end of the heavy chain peptide that was site-specifically biotinylated with EZ-Link® Maleimide-PEG_2-Biotin (Thermo Scientific) as described earlier [10].

Nanoparticle-bioconjugates

Primary amino groups of 8I7-Mab were covalently coupled to activated carboxyl groups of the nanoparticles using a procedure from Valanne et al. [17] with some minor modifications. Activation of 4.6×10^{12} particles was performed in 360 μL volume of 10 mmol/L phosphate buffer (pH 7.0) by applying sulfo-NHS and EDC to final concentrations of 10 mmol/L and 0.75 mmol/L, respectively. The antibodies (0.45 mg) were coupled to the activated particles in 900 μL volume of 10 mmol/L phosphate buffer (pH 8.0) containing 5.8 g/L NaCl. Final washes and blocking of the remaining active groups were performed in tris-based buffer (10 mmol/L tris, 0.5 g/L NaN_3, pH 8.5), and the nanoparticle-bioconjugates were stored in the same buffer supplemented with 2 g/L BSA at 4 °C. Before the first instance of use, the particles were mixed thoroughly, sonicated and centrifuged lightly (350 ×g, 5 min) to separate noncolloidal aggregates from the monodisperse suspension.

cTnI standards and plasma samples

Human cardiac troponin (native, tissue-derived cTnI–cTnT–troponin C (TnC) complex) was purchased from HyTest. Standards were prepared by diluting the troponin complex in TSA buffer (50 mmol/L Tris, pH 7.75, 9 g/L NaCl, and 0.5 g/L NaN_3) supplemented with 75 g/L BSA. Standards were stored at −20 °C so that a freshly thawed set of standards was used in every assay. Lithium heparin plasma from apparently healthy 32 volunteers (22 females/10 males; mean age 32; range 24–60 years) was collected at the Department of Biotechnology, University of Turku (Turku, Finland), and used in this study with the consent of the blood donors. All plasma specimens were stored at −20 °C until use. Prior to analysis, the frozen samples were thawed at ambient temperature, mixed and centrifuged (1 min, 2000 ×g) to remove any particulate material.

Nanoparticle-based immunoassays

The nanoparticle-based assay employed 19C7-Mab and one of the four different forms of antibody clone 9707 (Mab, $F(ab')_2$, Fab or cFab) as captures. The assay was performed in SAv-coated microtiter wells in one-step sandwich-type format. First, fixed amount of biotinylated capture antibodies (see Table 1) were immobilized to SAv-coated wells in 50 μL of Kaivogen buffer. After 1 h incubation at ambient temperature, the wells were washed twice with Kaivogen wash solution. Thereafter, 3.75×10^8 nanoparticle-bioconjugates in 40 μL of assay buffer and 10 μL of standard or plasma sample were applied to the capture wells and incubated for 15 min at +36 °C (650 rpm). The assay buffer (37.5 mmol/L Tris, pH 7.75, 30 g/L NaCl, 0.4 g/L NaN_3, 0.6 g/L BGG, 25 g/L BSA, 50 g/L D-trehalose, 0.8 g/L native mouse IgG, 0.05 g/L denatured mouse IgG, 2 g/L casein, and 37.5 IU/mL heparin) was modified from von Lode et al. [18]. Finally, the wells were washed thoroughly with Kaivogen wash solution and long life-time fluorescence of the bound nanoparticle-bioconjugates was measured directly from the surface in a time-resolved mode with Victor™ 1420 Multilabel Counter (PerkinElmer Life and Analytical Sciences, Wallac Oy, Turku, Finland) under pulsed ultraviolet excitation (excitation wavelength, 340 nm; emission wavelength, 615 nm; delay time, 400 μs; window time, 400 μs; cycle time, 1 ms; total measurement time, 1 s).

Comparison of captures and assay kinetics

The performance of different 9707 capture antibody forms was compared by measuring the cTnI concentration of 32 normal plasma samples. When testing assay kinetics with 100 ng/L endogenic cTnI, low cTnI concentration sample was spiked with a patient sample containing known concentration of cTnI. Sample signals were converted into cTnI concentrations obtained from a standard curve.

Table 1
Antibody amounts exploited in the four immunoassays.

9707		19C7	
Antibody form	ng/well	Antibody form	ng/well
Mab	100	Mab	100
$F(ab')_2$	66	Mab	100
Fab	16.5	Mab	25
cFab	16.5	Mab	25