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Enzymes and Protein Markers

Characterization of cardiac troponin subunit release into serum after acute myocardial infarction and comparison of assays for troponin T and I

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We examined the release of cardiac troponin T (cTnT) and I (cTnI) into the blood of patients after acute myocardial infarction (AMI). Three postAMI serum samples were applied in separate analytical runs onto a calibrated gel filtration column (Sephacryl S-200), and the proteins were separated by molecular weight. Using commercial cTnT and cTnI assays measured on collected fractions, we found that troponin was released into blood as a ternary complex of cTnT-I-C, a binary complex of cTnI-C, and free cTnT, with no free cTnI within the limits of the analytical methodologies. The serum samples were also examined after incubation with EDTA and heparin. EDTA broke up troponin complexes into individual subunits, whereas heparin had no effect on the assays tested. We added free cTnC subunits to 24 AMI serum samples and found no marked increase in the total cTnI concentrations, using an immunoassay that gave higher values for the cTnI-C complex than free cTnI. To characterize the cross-reactivity of cTnT and cTnI assays, purified troponin standards in nine different forms were prepared, added to serum and plasma pools, and tested in nine quantitative commercial and pre-market assays for cTnI and one approved assay for cTnT. All nine cTnI assays recognized each of the troponin I forms (complexed and free). In five of these assays, the relative responses for cTnI were nearly equimolar. For the remainder, the response was substantially greater for complexed cTnI than for free cTnI. Moreover, there was a substantial difference in the absolute concentration of results between cTnI assays. The commercial cTnT assay recognized binary and ternary complexes of troponin on a near equimolar basis. We conclude that all assays are useful for detection of cardiac injury. However, there are differences in absolute cTnI results due to a lack of mass standardization and heterogeneity in the cross-reactivities of antibodies to various troponin I forms.

Troponin is a regulatory protein of the thin filament of striated muscle, and consists of three subunits: C at 18 kDa, I at 21 kDa, and T at 37 kDa *(1)*. The cardiac forms of troponin T (cTnT)[6] and I (cTnI) are important serologic markers for diagnosis of acute myocardial infarction (AMI) *(2, 3)* and detection of minor myocardial injury *(4, 5)*. Unlike creatine kinase (CK) and the CK-MB isoenzyme, assays for cTnT and cTnI are highly specific to cardiac injury, with no increases observed in patients with skeletal muscle disease or trauma *(6, 7)*. The majority of troponin T and I proteins are located within the contrac-

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[6] Nonstandard abbreviations: cTnT, cardiac troponin T; cTnI, cardiac troponin I; AMI, acute myocardial infarction; CK, creatine kinase; cTnC, cardiac troponin C; DTT, dithiothreitol; and MOPS, 3-(*N*-morpholino) propane sulfonic acid.

tile apparatus of striated muscle. The concentration of these subunits is increased in blood for many days after AMI, because release from the structural elements requires degradation of the myofibril itself. In addition to the bound subunits, there are also free cytoplasmic components, estimated to be 6–8% for cTnT *(8)* and 3–4% for cTnI *(9)*. It has been thought that release of the cytoplasmic troponin accounted for the appearance of cTnT and cTnI during the initial stages of injury. Recently however, other forms of troponin have been shown to be released into the blood of individuals after AMI *(10, 11)*. The forms of troponin released include the complexes of troponin I, T, and C and the free subunits. In addition, cTnI was released as both oxidized and reduced forms, the oxidation being a result of intramolecular disulfide formation of two cysteines *(10)*. Moreover, cTnI contains a number of serine groups that are phosphorylated in cardiac tissues *(12)*. Human cTnT contains no cysteine group; therefore it is not capable of forming a disulfide bond.

The release of the troponin complex after myocardial injury has not been fully characterized. Using Western blot analysis, Lavigne et al. *(13)* suggested that cTnI occurs predominately as free subunits, with no evidence of a troponin I-T complex. In contrast, Katrukha et al. *(11)* used specific monoclonal antibodies and suggested that troponin I exists in blood predominately as the I-C complex, with <10% found in the free form. Morjana et al. *(14)* also concluded that serum of AMI patients contains cTnI complexes, along with partially degraded cTnI. There is also little data on the release of troponin T subunits into blood after AMI.

An understanding of the release pattern for troponin subunits after AMI is important when comparing results of different commercial assays for these proteins. The number of cTnI assays that are approved by the Food and Drug Administration increases each quarter. Unfortunately, there is no consensus of cTnI results because values from one assay to another can differ by a factor of 10 or more *(15)*. These discrepancies are largely due to differences in the reference materials used in the assay calibration. The lack of mass standardization also existed for CK-MB mass assays before the development of a CK-MB standard *(16)*. Unlike CK-MB, where nearly all commercial mass assays make use of the "Conan" antibody *(17)*, cTnI assays use different antibodies. Therefore differences in the antibody recognition of various troponin I forms are expected. In the current study, we used gel filtration chromatography to examine the troponin T and I forms released after myocardial injury. We also characterized the immunoreactive response of different commercial cTnI assays to purified troponin forms.

Materials and Methods

GEL FILTRATION ANALYSIS

A 90 cm × 2.6 cm column (Pharmacia Fine Chemicals) was packed with 700 mL of Sephacryl S-200 gel (Sigma Chemical Co.). The gel was prepared in an equilibration buffer, 50 mmol/L Tris-HCl, pH 7.5, containing 100 mmol/L KCl. The gel was suspended in ~2 volumes of buffer, allowed to settle (60 min), and the small particles were removed by decantation. This process was repeated three times. The column was calibrated for molecular weight, using commercial standards (Sigma) before the loading of each sample. The void volume was determined by blue dextran (M_r 2000). The other standards were β-amylase (M_r 200 000), bovine albumin (M_r 66 000), carbonic anhydrase (M_r 29 000), and cytochrome C (M_r 12 400). Purified ternary complexed and free troponin T and I subunits were obtained from Hytest Ltd. and were also used to calibrate the column. At the time these studies were performed, we did not have the binary complex of cTnI-C, thus we did not calibrate the column for this complex. To recover free troponin, cardiac troponin C (cTnC) was added to the collection tubes that corresponded to the expected elution tubes for cTnI, based on the known molecular weight for cTnI.

The protocol for use of human subjects was reviewed and approved by the Hartford Hospital Institution Review Board. Because leftover blood from routine collections was used for all samples and no patient identifiers were used, informed consent was deemed unnecessary. Three serum samples from patients with Q-wave AMI were separately added to the gel filtration column. A diagnosis of AMI was made by attending physicians, using standard World Health Organization criteria *(18)*, including the results of total CK and CK-MB. To ensure a high concentration of troponin subunits, the selected samples were collected 24–72 h after onset and were at or near the peak troponin values. The cTnI concentration for serum samples 1–3 were 200, 240, and 450 μg/L, respectively (Opus Plus, Dade Behring). Samples were loaded within 24 h after collection. One milliliter of serum was added to the column for fractionation. One-milliliter fractions were collected on an automated collector (Pharmacia), capped, and stored at 4 °C until analysis. In addition, 1.0 mL of sample 2 (serum) was added to a 3-mL heparin blood collection tube (final concentration, 15 units), and 1.0 mL of sample 3 was added to an EDTA blood collection tube [final concentration, 25 mg/L (2.5 mg/dL) of K_3EDTA]. These samples were incubated overnight and applied separately to the column. Collected fractions were assayed for cTnT, using the second generation assay on the ES300® (Roche Boehringer Mannheim), and assayed for cTnI, using the Opus Plus® and a prototype cTnI assay on the Immuno 1® (Bayer Diagnostics).

To further compare results of serum vs heparin-treated plasma, 50 samples, each simultaneously collected into a serum (red) and heparin-containing plasma (green) blood tubes, were assayed for cTnI, using the Opus Plus and Immuno 1. A linear regression analysis was performed on these data. Twenty-four serial serum samples from AMI patients were tested for cTnI, using the Opus Plus before and after the addition of cTnC to a final concentration of 100 μg/L. Some of these were also tested for cTnI on the

Opus Plus after addition of 10 μg/L of cTnC. Negative-troponin serum and heparin-treated plasma pools were prepared for use as matrices for the preparation of the troponin-supplemented samples. These pools consisted of blood from healthy individuals and were assayed for cTnT and cTnI to verify the low concentration before use. Three different high troponin serum pools were prepared from AMI samples. The pools were aliquoted and stored frozen at −70 °C before testing on selected cTnI analyzers capable of using serum as a specimen.

PREPARATION OF TROPONIN STANDARDS AND SAMPLES

All containers and pipettes that contacted cTnI and cTnT complexes were polypropylene. Three milligrams of lyophilized human cTnI (Bio-Tech International) at pH 3.0 was reconstituted in 1.5 mL of 1.0 mmol/L HCl. The reconstituted cTnI solution was centrifuged at 16 000*g* for 10 min to remove any particulate matter. The cTnI concentration in the supernatant was determined from the absorbance at 280 nm, using the equation: concentration (g/L) = A_{280}/0.44 *(19)*. A minor 3% correction for scattering was made to the value of A_{280} by extrapolation of the absorbance readings at wavelengths >350 nm. The cTnI concentration was 1.66 g/L. The absorptivity value was checked by calculation from the amino acid sequence of human cTnI as described by Pace et al. *(20)*. The calculated value of 0.41 was in good agreement with the experimentally determined value of 0.44 used in this work. The cTnI was diluted to a final concentration of 11.0 mg/L in a 3 mmol/L dithiothreitol (DTT, Pierce Chemical Co.), 50 mmol/L 3-(*N*-morpholino) propane sulfonic acid (MOPS; Fisher Biotech), 630 mmol/L NaCl, 40 g/L bovine serum albumin (Bayer Pentex) buffer, pH 7.10. DTT was used to reduce the intramolecular disulfide bond between the cysteine residues of the cTnI and to reduce any mixed disulfides formed between the cysteine residues and external reductant used during the purification of the protein. Four hours after the reduction reaction was started, the reduced sample was split into two aliquots: One (reduced troponin I) was diluted with deionized water to a final concentration of 10.3 mg/L; the other was oxidized by the addition of peroxide to a concentration of 20 mmol/L. Thirty minutes after the addition of peroxide, catalase (Calbiochem) was added to the oxidized sample to a final concentration of 1.4 mg/L to convert the hydrogen peroxide to oxygen and water. The final oxidized cTnI concentration was 10.3 mg/L. Three hours after the catalase was added to the oxidized sample, both the reduced and oxidized cTnI sample solutions were frozen in liquid nitrogen and stored at −70 °C.

Ternary troponin complex. Human cardiac ternary troponin complex (Bio-Tech) was obtained frozen in a buffer solution consisting of 60 mmol/L MOPS, 1.0 mol/L KCl, and 2 mmol/L $CaCl_2$, pH 7.2. The stated concentration of troponin complex was used to calculate the final molar concentrations of troponin I. The concentration was confirmed by measuring the absorbance at 280 nm (with a minor 9% correction for scattering made to the value of A_{280} by extrapolation of the absorbance readings at wavelengths >350 nm). The concentration was determined using the equation: concentration (g/L) = A_{280}/0.37. The found value was within 3% of the stated value. The absorptivity 0.37 was obtained by averaging the absorptivities of troponins I, C, and T. This coefficient was verified by the amino acid sequence of human cardiac troponin complex *(21)*. The calculated value of 0.41 is in good agreement with the experimentally determined value of 0.37 used in this work. The ternary troponin complex was diluted to a final troponin I concentration of 10.7 mg/L in a buffer consisting of 40 mmol/L MOPS, 2 mmol/L $CaCl_2$, 40 g/L bovine serum albumin, and 600 mmol/L NaCl, pH 7.1. Aliquots of the troponin complex were frozen in liquid nitrogen and stored at −70 °C.

cTnT. Lyophilized human cTnT (0.10 mg; Scripps Laboratories) was reconstituted with 0.40 mL of 10 mmol/L HCl. The cTnT was diluted to a final concentration of 15.0 mg/L in a 50 mmol/L MOPS, 630 mmol/L NaCl, 2 mmol/L $CaCl_2$, 40 g/L bovine serum albumin (Bayer Pentex) buffer, pH 7.10. Aliquots of cTnT were frozen in liquid nitrogen and stored at −70 °C. cTnT does not contain any cysteine residues, and therefore does not undergo oxidation/reduction.

Binary complexes. Frozen aliquots of oxidized or reduced cTnI at 10.3 mg/L and cTnT at 15.0 mg/L were thawed at room temperature. For the I-C complex, lyophilized human cTnC and $CaCl_2$ were added directly to the oxidized or reduced cTnI solutions to obtain final concentrations of 10.3 mg/L cTnI, 10.4 g/L cTnC, and 1 mmol/L $CaCl_2$. For the I-T complex, the cTnI solutions were mixed in equal volumes with cTnT to obtain final concentrations of 5.15 mg/L cTnI and 7.5 mg/L cTnT. For the T-C complex, lyophilized cTnC was added directly to cTnT to obtain final concentrations of 15.0 mg/L cTnT and 15.2 g/L cTnC. All solutions were incubated 2 h after the components were mixed, and then were frozen in liquid nitrogen and stored at −70 °C. From these stock preparations, dilutions were aliquoted into troponin-free serum and plasma pools to produce working samples at three different troponin concentrations. Table 1 lists the composition and concentrations of the working troponin samples used in the comparative study. To minimize air oxidation, samples were individually prepared and frozen to −70 °C within 10 min after preparation.

INTERASSAY COMPARISONS FOR cTnI

Nine cTnI assays were tested using the working troponin samples described above. The identities of the specific assays were not revealed. The commercial assays in-

Table 1. Composition and expected concentration (μg/L) of troponin T and I samples prepared in pooled serum and used in the comparison of assays.

	High		Mid		Low	
Sample composition	**cTnI**	**cTnT**	**cTnI**	**cTnT**	**cTnI**	**cTnT**
Oxidized free cTnI	41.4	0	10.3	0	5.1	0
Reduced free cTnI	41.4	0	10.3	0	5.1	0
Ternary cTnT-I-C complex	42.8	61.6	10.7	15.4	5.3	7.7
Binary cTnI-C complex (oxidized cTnI)	41.4	0	10.3	0	5.1	0
Binary cTnI-C complex (reduced cTnI)	41.4	0	10.3	0	5.1	0
Free cTnT	0	45.0	0	15	0	7.5
Binary cTnT-C complex	0	45.0	0	15	0	7.5
Binary cTnT-I complex (oxidized cTnI)	20.8	30.0	5.2	7.5	2.6	3.7
Binary cTnT-I complex (reduced cTnI)	20.8	30.0	5.2	7.5	2.6	3.7

cluded the Opus Plus, Access® (Beckman), Stratus II® (Dade Behring), AxSYM® (Abbott Laboratories), Immuno 1 (Bayer Diagnostics), Triage® Cardiac System (Biosite Diagnostics), and Alpha Dx® (First Medical). The prototype cTnI assays were the Immulite® (Diagnostic Products) and the ACS:180® (Chiron Diagnostics). Serum was used in all assays except for the Triage device, for which heparin-treated plasma was used. Serum was also tested for cTnT, using the ES300. To minimize air oxidation of cTnI, each of the 27 samples listed in Table 1 was assayed within 1 h after it was thawed at room temperature.

Results

GEL FILTRATION ANALYSIS

Fig. 1 illustrates the retention time for the molecular weight standards, purified proteins for free troponin T and I, and the ternary complex. The absolute retention times varied slightly from run to run because of changes in analysis conditions. We estimated the accuracy of the molecular weight determination to be ~10%.

Fig. 2 illustrates the gel filtration results for AMI serum sample 1. For cTnT, there were three peaks that have molecular weights that corresponded to a troponin ternary complex of T-I-C, free troponin T, and immunoreactive fragments (Fig. 2A). When these fractions were tested for cTnI (Opus Plus), only two peaks were evident, corresponding to the ternary complex and the binary complex of I-C (Fig. 2B). No free cTnI was observed. When the serum sample was incubated with EDTA, the results showed a disappearance of the ternary complex for both cTnT (Fig. 2C) and cTnI (Fig. 2D). The absolute concentrations for each band was also markedly decreased. Similar results were also observed for AMI sample 3 (results not shown). We also tested fractions collected from sample 1 for cTnI, using a prototype Immuno 1 assay, which produced similar results to those shown in Fig. 2, A-D (data not shown).

Figure 3 illustrates results for AMI sample 2. In contrast to the other two AMI samples, this sample did not contain any major peaks corresponding to the ternary troponin T-I-C complex, but did contain the cTnI-C complex (Fig. 3, A and B). Instead, very low molecular weight cTnI fragments were observed that were not seen in either of the other two samples (Fig. 3B). Sample 2 was also tested after incubation with heparin (Fig. 3, C and D). There was no substantial difference in the release pattern

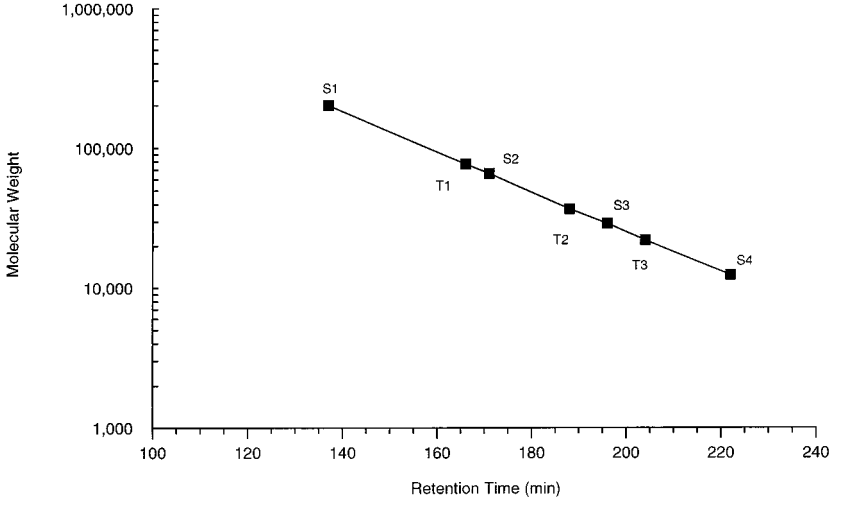

Fig. 1. Calibration curve for gel filtration column using molecular weight standards and purified troponin T and I subunits.

Sigma standards: S1, amylase (M_r 200 000); S2, albumin (M_r 66 000); S3, carbonic anhydrase (M_r 29 000); S4, cytochrome C (M_r 12 400). Troponin standards: T1, cTnT-I-C complex (77 kDa); T2, free cTnT (37 kDa); T3, free cTnI (22 kDa).

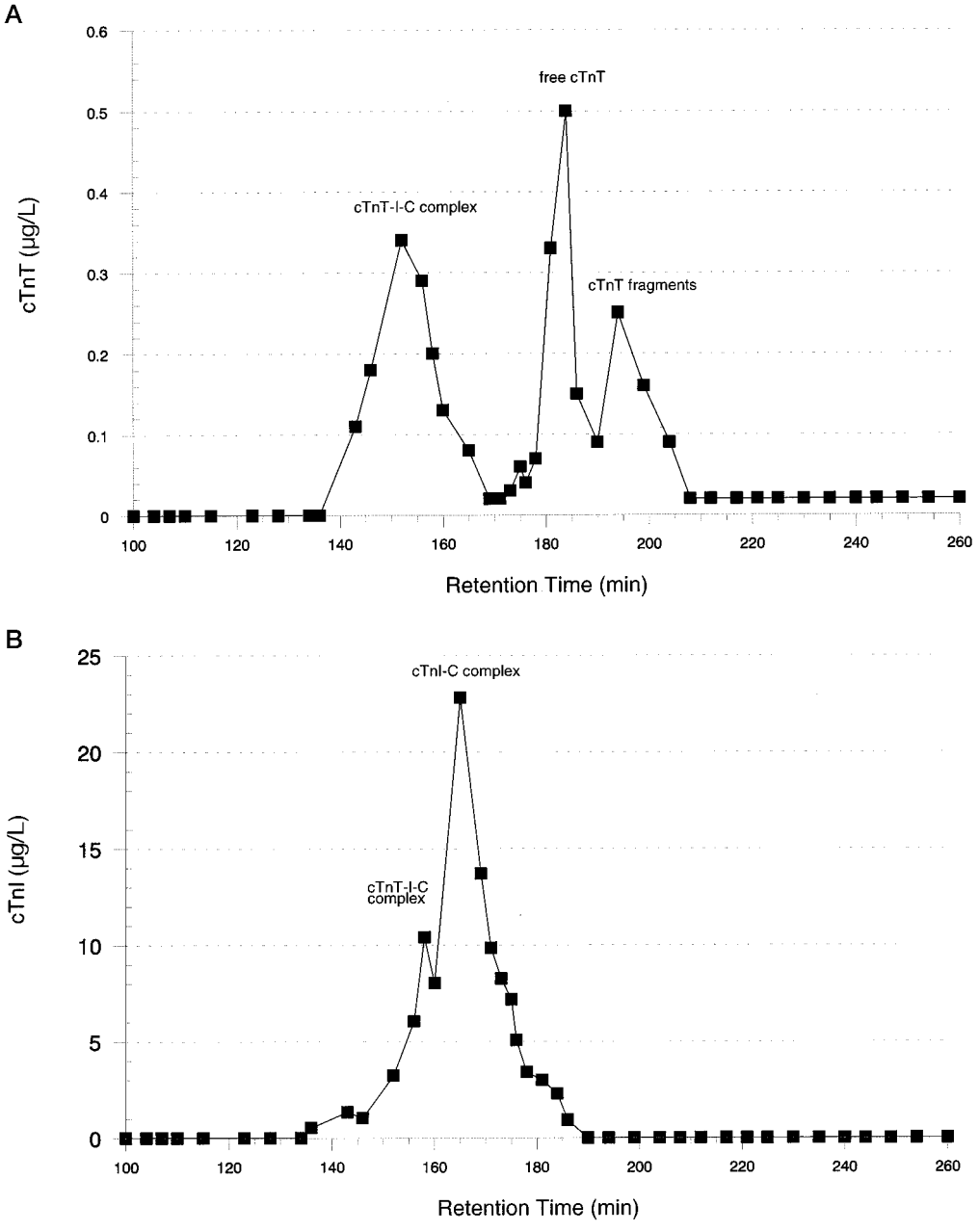

Fig. 2. Elution curve for AMI patient 1 (cTnI = 200 μg/L).

(*A*) Collected fractions assayed for cTnT; (*B*) fractions assayed for cTnI (Opus Plus); (*C*) serum incubated with EDTA and collected fractions assayed for cTnT; and (*D*) serum incubated with EDTA and fractions assayed for cTnI.

of troponin subunits, and the recovered troponin subunit concentrations were within experimental limits. The effect of heparin on the ternary complex could not be determined because this particular sample did not have any of this complex. The results for cTnI, using the Immuno 1 assay showed identical results (data not shown).

The results comparing serum and heparin-treated plasma samples simultaneously collected on AMI and non-AMI patients are shown in Fig. 4, A (Opus Plus) and B (Immuno 1). The linear regression showed no marked difference between serum and heparin-treated plasma, confirming the results from the gel filtration experiments.

Free cTnC at 100 μg/L and 1 mg/L was added to serum tubes from AMI patients before testing for cTnI, in an attempt to recover any free cTnI that might have been lost to plastic or glass surfaces during the measurement procedure. The mean (± standard deviation) for the 24 samples was 29.3 (± 37.0) μg/L without addition of cTnC, and was not different from the result of 29.7 (± 36.7) with addition of 100 μg/L. On a subset of seven samples, the

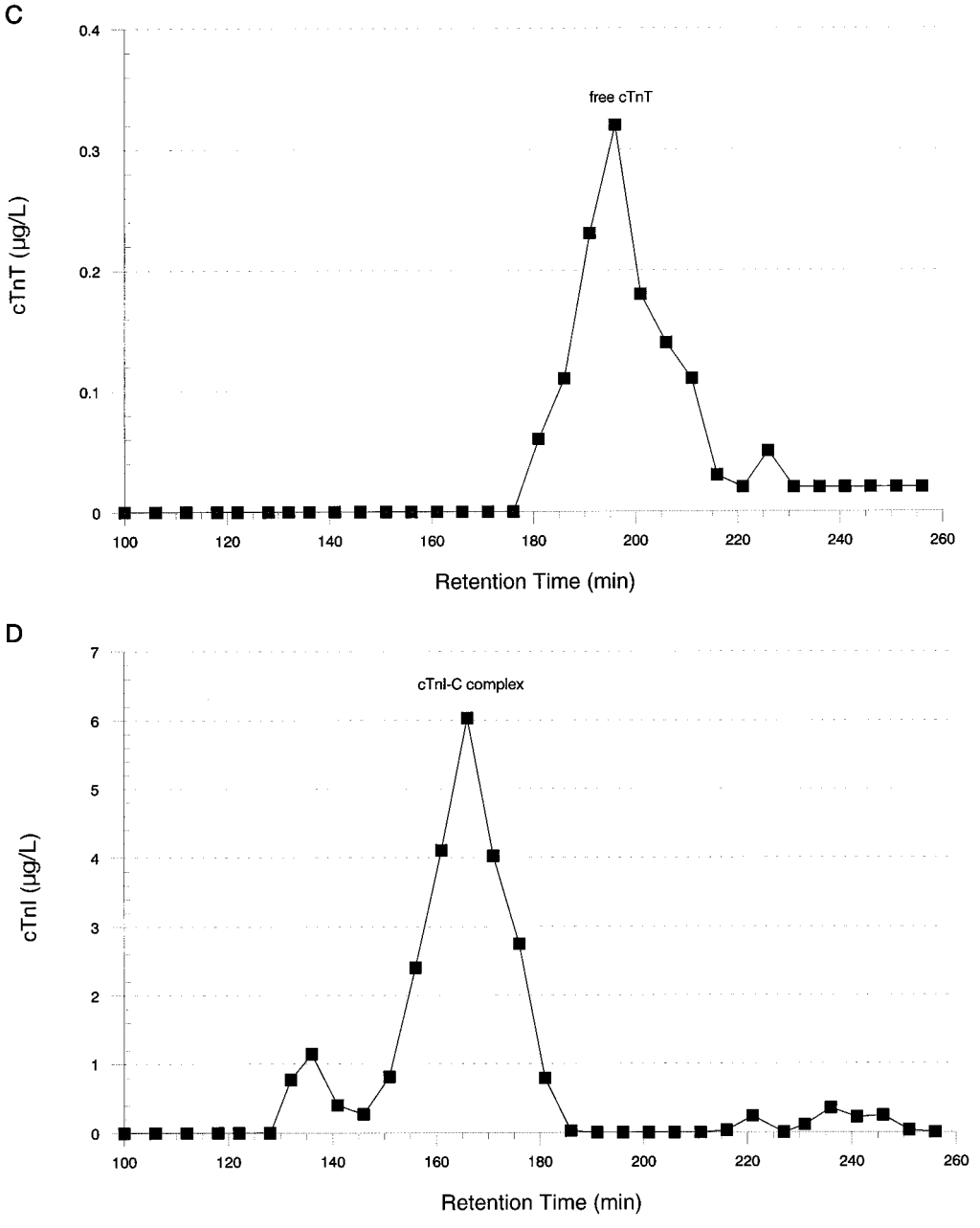

Fig. 2, continued.

mean (± SD) for cTnI without and with 1 mg/L of cTnC was also not different at 27.6 (± 23.6) and 27.7 (± 22.9) µg/L, respectively.

INTERASSAY COMPARISON OF cTnI RESULTS

The results of the interassay comparison of cTnI assays are illustrated in Fig. 5A. All results are plotted on a relative scale, according to the expected values shown in Table 1. None of the cTnI assays responded to free cTnT or the binary complex of cTnT-C, and thus these results are not shown. All of the assays tested were sensitive to each of the prepared samples containing cTnI subunits. There were differences in the absolute and relative responses to each other. Assays 2, 5, and 7 had roughly equimolar responses for each of the seven cTnI forms and produced results that were about 70–110% of the expected concentrations listed in Table 1. Assays 4, 8, and 9 also produced reasonably equimolar responses, but at a concentration that was ~2.5-fold, 4-fold, and 4.5-fold those listed in Table 1, respectively. The response for assay 3 was similar to assays 2, 5, and 7 for free cTnI (oxidized and reduced) and binary cTnI-T complexes; however, for the ternary and binary cTnI-C complexes, results were about threefold those of the free cTnI. Assays 1 and 6, in general, also showed higher responses for the ternary complex and binary cTnI-C complexes. In addi-

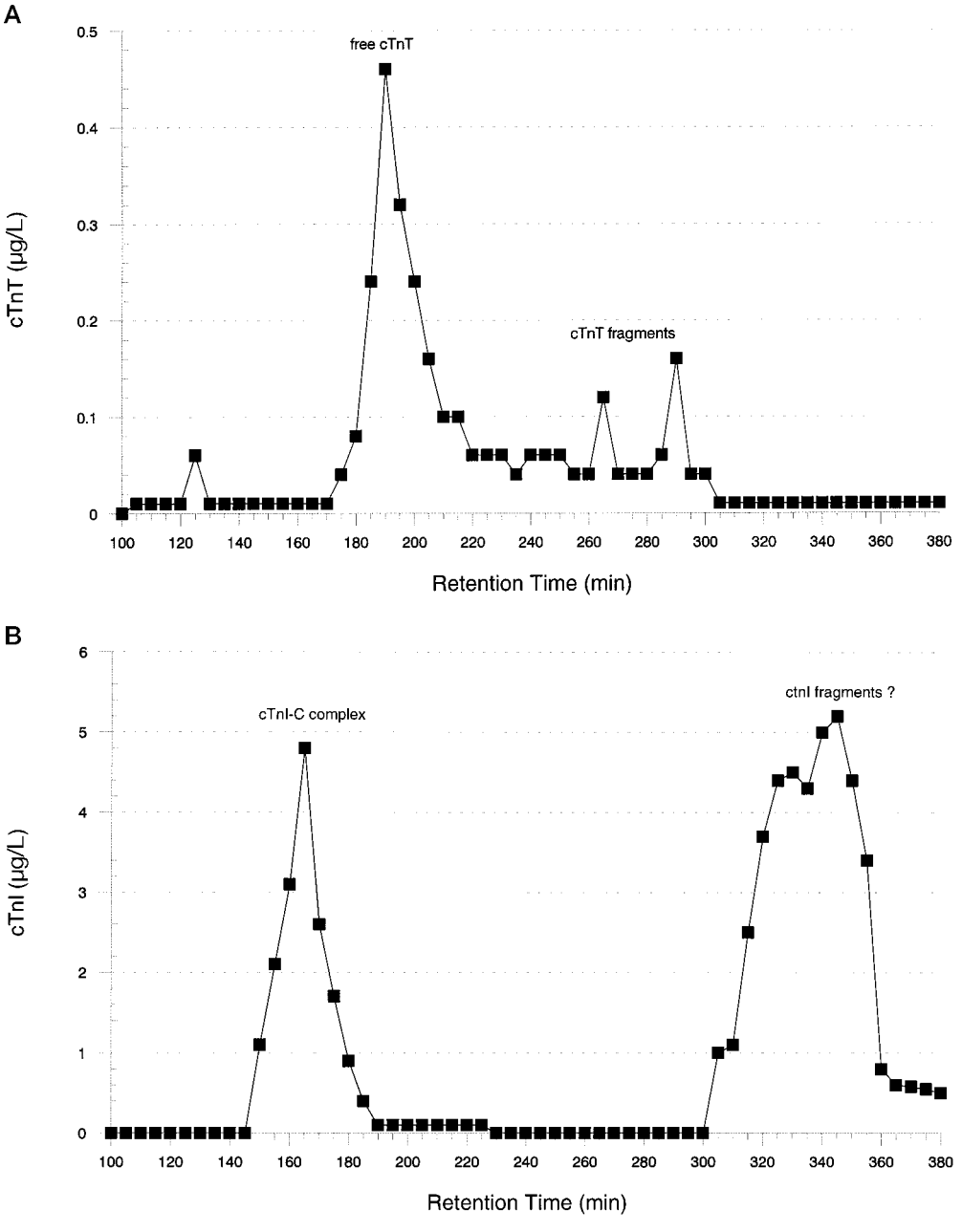

Fig. 3. Elution curve for AMI patient 2 (cTnI = 240 μg/L).

(*A*) Collected fractions assayed for cTnT; (*B*) fractions assayed for cTnI (Opus Plus); (*C*) serum incubated with heparin and collected fractions assayed for cTnT; and (*D*) serum incubated with heparin and fractions assayed for cTnI. It is unknown if cTnI fragments are released or if they are higher molecular weight complexes or free subunits that eluted at a later time.

tion, each of these latter assays had nonequal responses with respect to oxidized and reduced cTnI, particularly when these cTnI subunits were part of complexes.

The comparison of serum and heparin-treated plasma samples for cTnI, using assay 4, is illustrated in Fig. 5B. Minor differences were observed for the binary I-C complexes, whereas major differences were observed for the ternary complex, the binary I-T complex, and free troponin I forms. The comparative cTnT results of purified samples for free cTnT, ternary cTnT-I-C complex, and the cTnT-C binary complex are shown in Fig. 5C. The cTnT assay did not produce positive results for free cTnI or either of the I-C binary complexes (thus results are not shown). The assay has a rather equimolar response to the ternary and binary complexes, which was about threefold higher than the response for the free cTnT form.

The results of the serum pool, using the different cTnI assays, are shown in Fig. 6. Results are plotted against assay 3. There was a high degree of analytical correlation for these three samples as shown. Assay 2 produced the

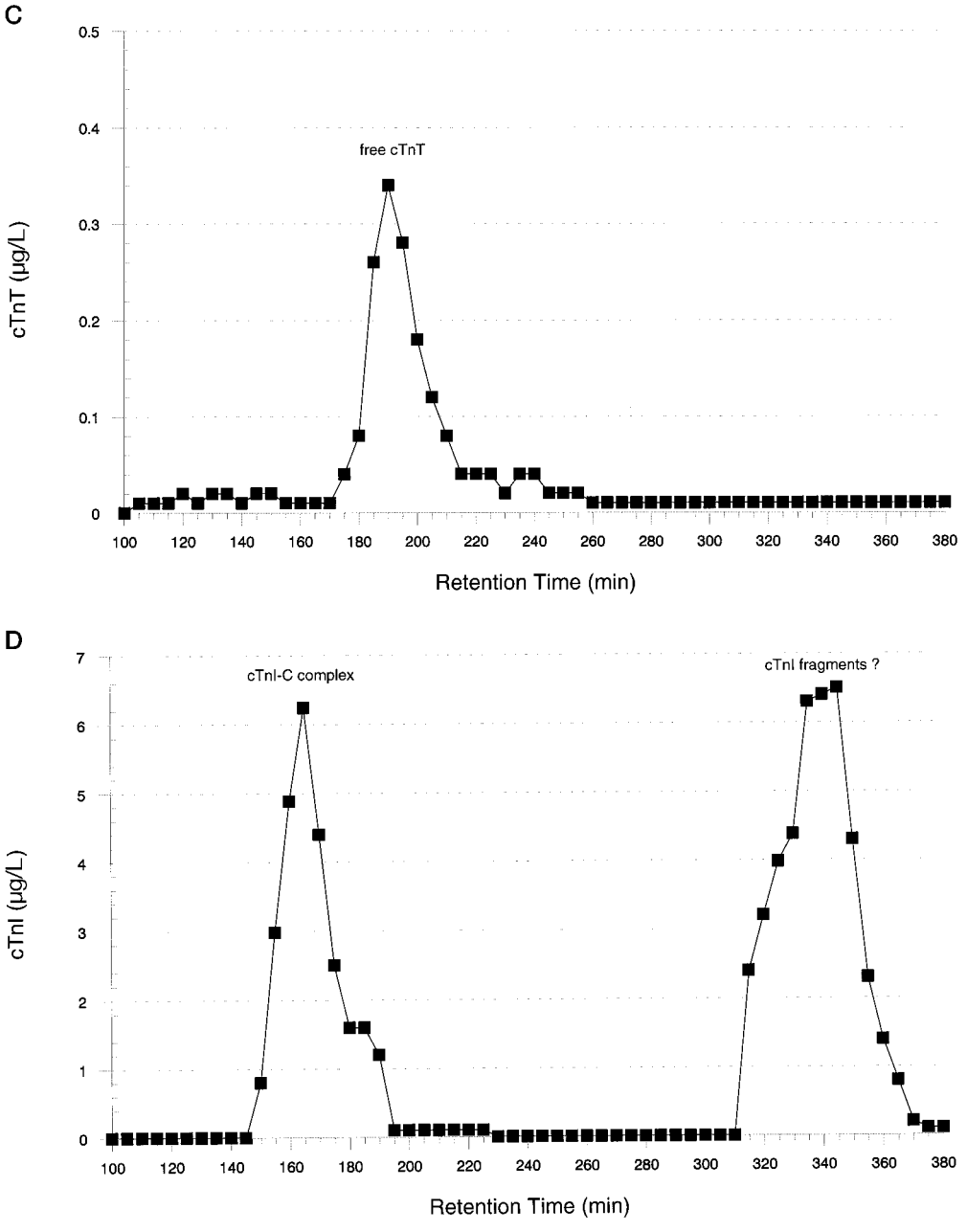

Fig. 3, continued.

lowest result, whereas assay 6 produced the highest result. The difference between the two extreme assays is roughly 20-fold.

Discussion

The primary function of measuring cardiac markers in blood is to detect the presence of myocardial injury. This study shows that all of the commercial and prototype troponin assays fulfill this requirement, because they all react to the appropriate forms that exist in blood after injury. There were differences, however, in the absolute and relative responses of specific assays to the various forms. The data presented in the gel filtration experiments essentially confirms the conclusions made by others *(10, 11)*, in that cTnI is released predominately in the complexed form, although some free cTnI was found. The correlation results of Fig. 6 also indirectly support our suggestion that there is little free cTnI in blood. For example, the results for complexed cTnI on assay 3 are 80% of those for assay 4 (Fig. 5A), and are consistent with the correlation observed by these two assays for the pooled AMI serum samples (slope, 0.90; Fig. 6). For free cTnI, however, Fig. 5A shows that assay 4 produced two- to threefold higher concentrations than assay 3. If real

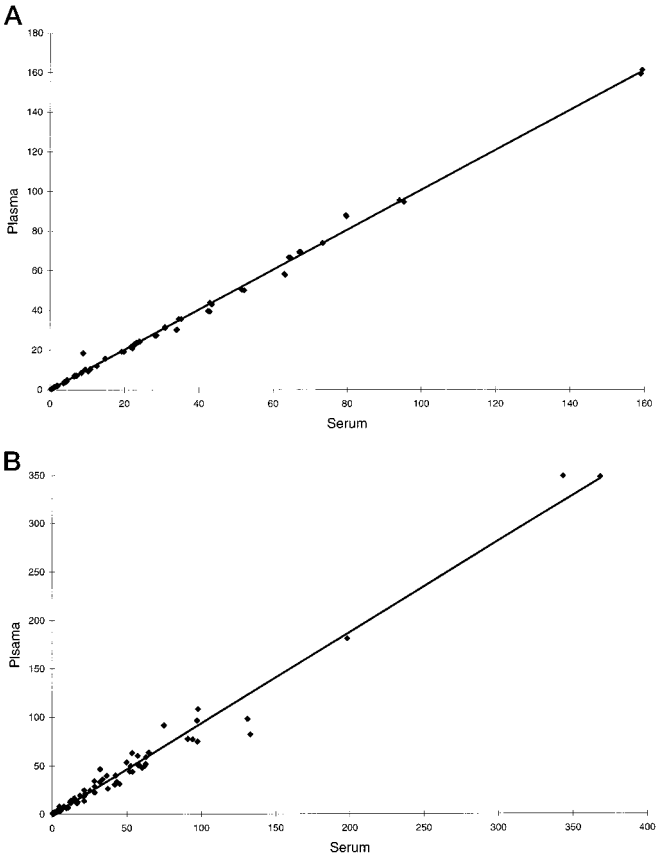

Fig. 4. Correlation between samples simultaneously collected in serum and heparin-treated plasma and assayed for cTnI, using (*A*) Opus Plus: y (plasma) = 0.943x (serum) − 0.726, r = 0.9900; or (*B*) Immuno 1: y = 1.009x − 0.0201, r = 9976.

serum samples had contained a high concentration of free cTnI, the slope of the correlation curve between assays 3 and 4 from Fig. 6 would have reflected this difference more closely. Fig. 5B also suggests that the predominate form must be the binary cTnI-C complex, because more differences in the serum/plasma correlation curves (Fig. 4) would be expected if free cTnI forms were a major fraction of samples after AMI. Similar serum/plasma comparisons were observed for cTnI assays 1 and 3. The data that shows that EDTA disrupts troponin complexes was expected because the affinity constant for the complex is decreased with the chelation of calcium *(22)*.

Our findings are consistent with results of the College of American Pathologists proficiency surveys for cTnI *(23)*. In the 1997 survey, laboratories (n >600) assayed samples, using the Access, Opus Plus, or Stratus II. The mean cTnI concentration varied substantially, depending on which cTnI assays was used. The Access assay produced the lowest concentrations, some 20-fold lower than results from the Opus Plus. Results between these extremes were reported for the Dade Stratus II, at about one-third of the Opus Plus assay.

The gel filtration study was very limited, in that only three serum samples were tested and that they all contained very high cTnI concentrations. We were not able to examine how troponin subunits are differentially released as a function of time after onset of injury. The gel filtration technique is very insensitive because of the dilution of fractions by the elution buffer; therefore, early and very late AMI samples with low troponin concentrations could not be tested without concentrating the eluates. It is likely that the pattern of release varies over time, possibly with intact ternary complexes predominating early, followed by binary complexes, free forms, and immunoreactive fragments. Katrukha et al. *(11)* showed that free cTnI was not a major component of total cTnI when blood was tested up to 100 h after AMI. We were also not able to verify the identity of the individual peaks as they eluted from the column. Variations in the column retention times of complexed and free subunits can affect interpretations. Some of the low molecular weight components shown in Fig. 3, B and D, may in fact be cTnI components that were retained by the column because of the adhesiveness of cTnI.

The disposition of free cTnI after release from damaged myocytes is unknown. The free subunit does have a high affinity for troponin C ($k = 1.27 \times 10^8$) *(22)*, and there may be binding with residual serum troponin C *(10)*. Because skeletal muscle troponin C is homologous to the cardiac subunit, there is probably some troponin C available in serum to bind cTnI *(10)*. Whether there is enough troponin C present to bind all the available free cTnI that is released into blood is unknown. There are no commercial assays for TnC, and a serum reference range has not been established. A prototype assay for fast skeletal muscle troponin I has been developed, and the reference limit was 0.2 μg/L *(24)*. The skeletal muscle troponin C concentration may be in this same range. CTnI can bind to other proteins present in blood, such as calmodulin, a structural analog to troponin C *(25)*, which may obscure antibody recognition sites. It may also be possible that free cTnI is lost to the surfaces of blood collection tubes, pipette tips, sample cuvettes, and so forth. It is also possible some free cTnI is released into blood but is not detectable by our gel filtration system because of its insensitivity to low concentrations or because of the manner in which the gel filtration experiments were conducted.

The absolute and relative differences between cTnI assays remain a concern for laboratories that switch from one assay to another as new instrumentation becomes available, or when a clinician attempts to compare results from one laboratory with another. The American Association for Clinical Chemistry cTnI Standardization Committee will be specifying a reference material based on a complex (binary or ternary) to be used as a primary standard. When the standard becomes available, manufacturers of cTnI assays can calibrate their assays to it, thereby greatly reducing the interassay variability of results. However, the data presented in Fig. 5A shows that interassay biases will not be entirely removed because each assay has different relative reactivities to the

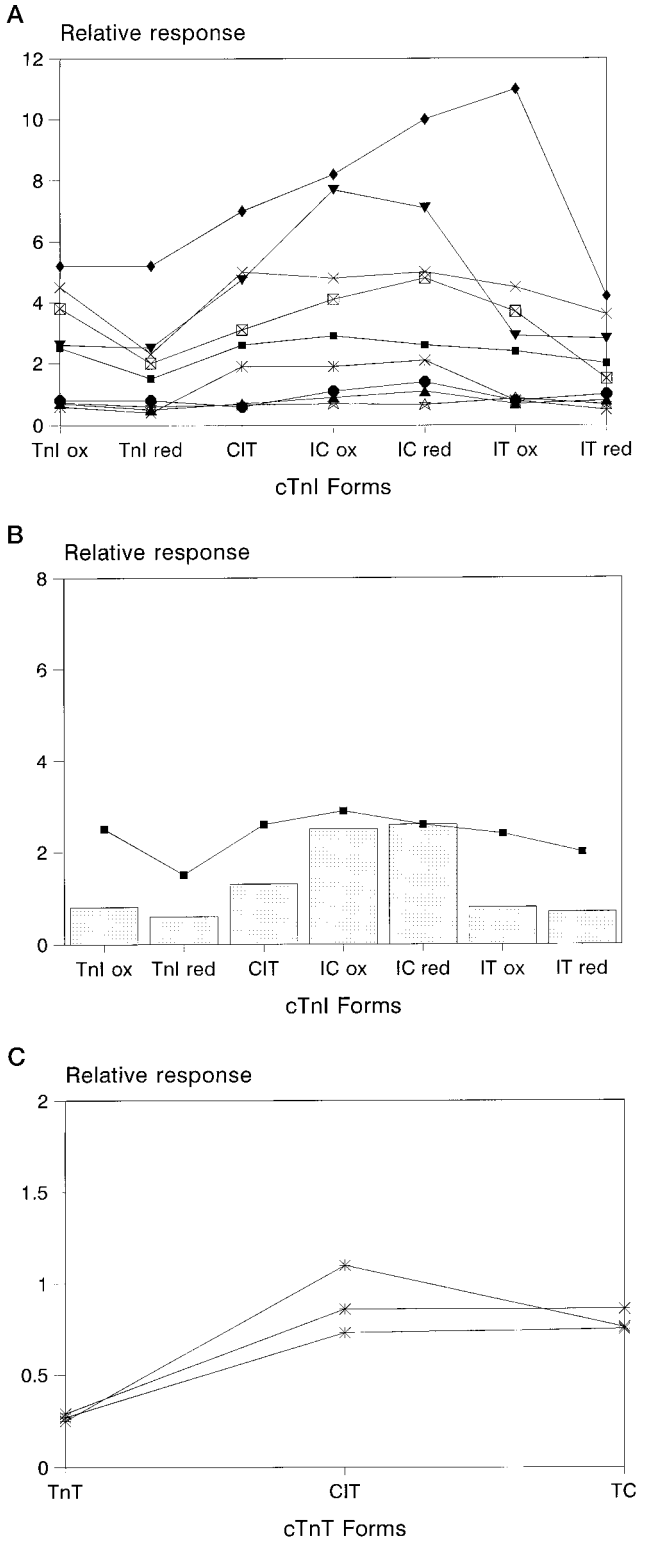

Fig. 5. Relative response for troponin assays to prepared samples of cTnI.

(*A*) cTnI in serum. Each point represents an average of results from three concentrations. Results are plotted on a relative scale, based on the expected concentrations listed on Table 1. (▼) Assay 1, (☆) assay 2, (*) assay 3, (■) assay 4, (●) assay 5, (♦) assay 6, (▲) assay 7, (☒) assay 8, (x) assay 9. (*B*) cTnI for serum (■) vs plasma (*bar*) for assay 4. (*C*) cTnT for serum. Each of the three concentrations for each form is presented.

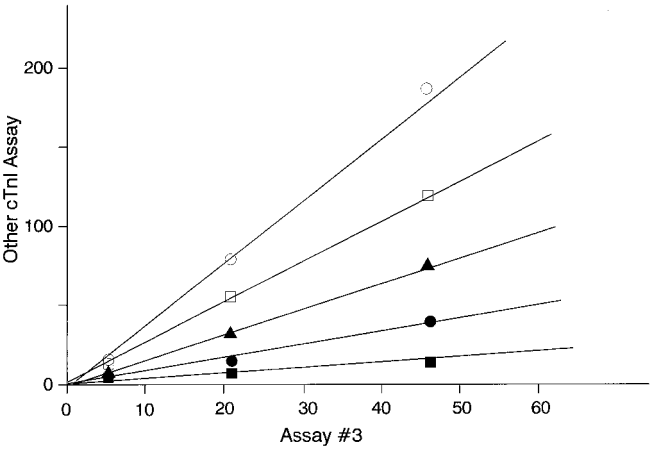

Fig. 6. Correlation of cTnI assays for a serum pool against assay 3.

(○) Assay 6, $y = 4.2x - 6.9$ μg/L, $r = 0.999$; (□) assay 1, $y = 2.6x - 2.3$ μg/L, $r = 0.9998$; (●) assay 4, $y = 0.90x - 1.4$ μg/L, $r = 1.000$; (■) assay 2, $y = 0.22x - 2.2$ μg/L, $r = 0.9804$; (▲) assay 9, $y = 1.68x - 1.29$, $r = 0.999$.

various cTnI forms. True interchangeability of results between cTnI assays will only occur if all manufacturers also standardize the antibodies used.

Differences in the reactivities between oxidized and reduced forms may also present stability problems for some assays. cTnI released from damaged tissue in the reduced form may oxidize with exposure to air within 5 h *(10)*. The oxidation can be accelerated with the addition of peroxide or inhibited with thiol-reducing agents such as 2-mercaptoethanol or DTT *(10)*. Unless stabilizing reagents are added, cTnI assays that exhibit a differential response to reduced vs oxidized forms (e.g., assay 6 of Fig. 5A) may exhibit changing results during the first few hours after blood collection, as cTnI slowly oxidizes to form disulfide linkages. In addition, the oxidation of cTnI in circulating blood will yield a differential response among assays that do not recognize the oxidized and reduced forms equally. We were not able to verify which cTnI assays demonstrated different results when samples are assayed immediately and after several hours of room temperature storage.

The analysis and standardization for cTnT is more straightforward than for cTnI. Because of patent rights, all commercial assays for cTnT are marketed by one manufacturer. Available laboratory-based and point-of-care testing devices are calibrated to the same reference material. The Elecsys® analyzer was not available to us; however, the same antibodies are used in all cTnT assays, and no difference in the results was expected. Free cTnT is recoverable in substantial concentrations in blood after injury. The analysis of free cTnT may offer additional clinical information over that of total cTnT. The low (or absent) concentration of free cTnI in the limited samples examined by this study may explain differences observed in the kinetics of cTnT vs cTnI release after AMI.

In conclusion, all cardiac troponin assays do a good job at detecting myocardial injury, irrespective to the absolute

value obtained. Because of the specificity of troponin, an abnormal blood concentration has clinical significance for cardiac disease.

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