Design and Analytical Evaluation of an Immunoassay for Long Forms of Cardiac Troponin T

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Background: Cardiac troponins (cTn) are used as diagnostic biomarkers of acute myocardial infarction (MI), but elevated levels of cTn can also be observed in other conditions. We report here the design and analytical evaluation of an immunoassay that targets intact and mildly fragmented forms of cardiac troponin T (referred to as long cTnT), which has been shown to better differentiate between MI and end-stage renal disease than the current Roche Elecsys® high sensitivity cTnT assay.

Methods: The long cTnT assay was evaluated for analytical characteristics. Serum and heparin plasma sample matrices were compared and the analyte stability was studied by storing endogenous long cTnT from samples of ST-segment elevation MI patients in heparin plasma or buffer at different temperatures and subjecting samples to freeze–thaw cycles. The correlation of long cTnT levels and time after MI symptom onset was also studied.

Results: The long cTnT assay achieved a limit of detection of 10.8 ng/L and a lower limit of quantitation (10% CV) of 30.8 ng/L. The response was linear from 5 to 5000 ng/L. Serum produced significantly lower results than heparin plasma. Endogenous long cTnT tolerated freeze–thaw cycles, but stability was compromised when stored at higher temperatures. The fraction of circulating long cTnT was highest during early hours of MI.

Conclusion: The long cTnT assay presented good analytical performance. Our results support using heparin plasma as the sample material and avoiding prolonged sample storing at room temperatures. Long cTnT fraction decreases in time after the onset of type 1 MI.

ClinicalTrials.gov registration: NCT04465591

INTRODUCTION

Cardiac troponins (cTn) I and T (cTnT) are measured as a part of the routine diagnostic workup in patients presenting with symptoms consistent with possible myocardial infarction (MI) in hospital emergency departments. After the introduction of high-sensitivity cTn tests (hs-cTn) and low cut-off limits, it has become increasingly more apparent that the clinical specificity of cTn for the diagnosis of MI is suboptimal, especially at lower concentrations. Minor chronic or temporary cTnI and cTnT

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IMPACT STATEMENT

Cardiac troponins (cTn) are the preferred diagnostic biomarkers for myocardial infarction (MI), but with current commercial cTn assays, non-MI related cTn elevations may be observed. Immunoassays targeting certain forms of cTn may offer better clinical specificity. In this study we present the design and analytical evaluation of an assay targeting long forms of cTnT that has previously demonstrated excellent discrimination between MI and renal patients. We also report important findings on the stability of long cTnT forms. Information described in this article offers insights to long cTn assay design and provides valuable knowledge of long cTnT as a biomarker.

elevations can be observed in multiple conditions that are not linked to MI such as myocarditis, chronic kidney disease, infectious diseases, or strenuous exercise (1,2). Distinguishing between non-ST elevation myocardial infarction (NSTEMI) and other non-MI conditions associated with elevated cTn can be challenging with the hs-cTn tests in contemporary clinical practice. Thus, increasing interest has arisen for assays targeting specific forms of cTn, which may offer better clinical specificity for type 1 MI (3,4).

CTnT is known to be present in the circulation of type 1 MI patients as fragments of various sizes. A higher proportion of intact or slightly fragmented cTnT (29–37 kDa) particles can be detected shortly after type 1 MI due to fresh cTnT release into circulation (5)—a sign of ischaemic damage and necrosis of the myocardium. The proportion of smaller fragments increases later after type 1 MI, as the cTnT molecule is broken down into smaller fragments (5,6). The main cleavage sites in published studies have been identified as between amino acid residues (aar) 68–69 and in the C-terminal cleavage area 189–223 (6,7). Thus, the main circulating cTnT forms in the presence of type 1 MI comprise intact cTnT (37 kDa), the N-terminally truncated form (29 kDa, aar 69–297), and the N- and C-terminally truncated central forms (~16 kDa) (7,8). However, in patients with chronic kidney disease and in marathon runners, the elevated hs-cTnT concentrations comprise more exclusively the small central fragments of cTnT (9–11).

Elecsys® hs-cTnT tests (Roche Diagnostics GmbH) are designed to detect the stable central part of cTnT (8,12). Thus, with Elecsys hs-cTnT tests the intact, N-terminally and C-terminally truncated forms are all measured, and therefore this result is denoted as "total cTnT" (Fig. 1). To enhance the study of cTnT fragmentation in terms of throughput and sensitivity, an immunoassay was developed to target the intact or mildly fragmented forms of cTnT that are not cleaved at the C-terminal cleavage area (Fig. 1).

Results obtained with our long cTnT assay have shown that the ratio between long cTnT and total cTnT can effectively distinguish type 1 MI patients from end-stage renal disease patients (13). The Elecsys hs-cTnT assay alone was not able to discriminate between these groups, as similar cTnT levels were seen in both groups. Here we report the design and analytical evaluation of our long cTnT immunoassay. In addition, data is provided on the stability of long cTnT in vitro and on the levels of long cTnT, total cTnT, and the long cTnT/total cTnT ratio in respect to the onset of symptoms.

MATERIALS AND METHODS

Long cTnT Assay Design

The novel long cTnT assay follows the sandwich-type immunoassay format and utilizes time-resolved-fluorescence for signal detection.

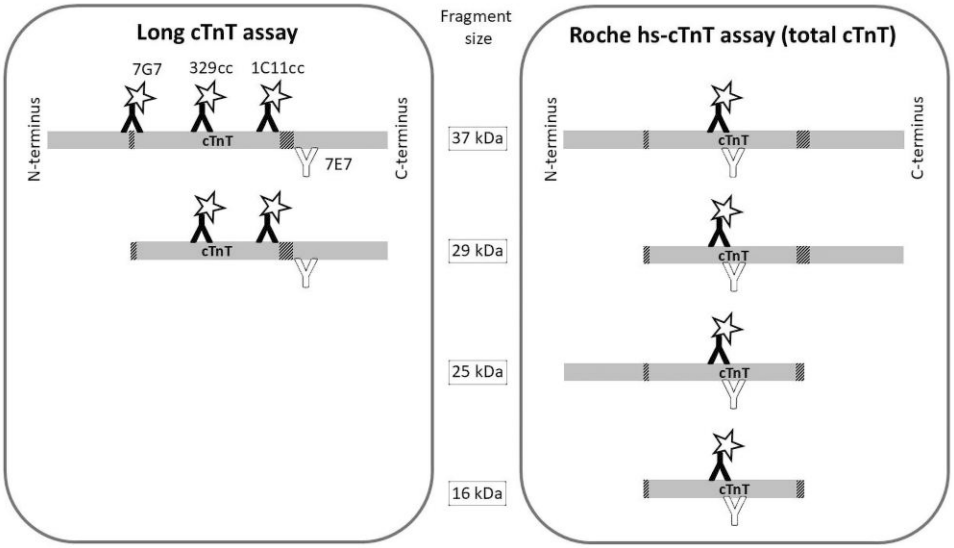

Fig. 1. Schematic representation of the main molecular forms of cTnT detectable by the long cTnT assay and the Elecsys hs-cTnT assay. The black tracer antibodies are marked with stars, while capture antibodies are white. The striped sections of cTnT indicate major cleavage sites (aar 68–69, 189–223). Fragmentation at the C-terminal cleavage area (aar 189–223) is being probed with the long cTnT assay.

The assay is based on the use of a monoclonal capture antibody (mab) targeting the C-terminal part of cTnT (mab 7E7, aar 223–242 according to UniProt entry P45370-6, HyTest Ltd.) and a combination of monoclonal tracer antibodies targeting cTnT parts toward the N terminus (mab 7G7, aar 67–86; mab 329 cc, aar 119–138; mab 1C11 cc, aar 171–190, all from HyTest) (Fig. 1). The reactivity toward certain parts and thus certain forms of cTnT of these antibodies has been confirmed by Western blot studies (6). The selected antibody combination allows the detection of the cTnT forms that are not cleaved in the C-terminal cleavage area at aar 189–223, i.e., the intact form of cTnT and N-terminally truncated forms. The use of multiple tracer antibodies with separate epitopes allows enhancement of the signal from single cTnT molecule and, with the selected epitopes, especially from the intact cTnT. Human cardiac troponin ITC-complex (HyTest) was used as a calibrator for the immunoassay as diluted in Tris-buffered saline with azide (TSA)-bovine serum albumin (BSA) buffer (tris-HCl [50 mmol/l] pH 7.75; NaCl [150 mmol/l]; NaN_3 [0.5 g/l]; bovine serum albumin [75 g/l]) (Probumin, Merck Millipore) and stored at −20°C until use.

Antibody Conjugation with Biotin or Europium Chelate for Long cTnT Assay

The capture antibody (7E7) was conjugated with 30-fold molar excess of biotin isothiocyanate (Biotechnology, University of Turku) in a sodium carbonate-bicarbonate buffer (50 mmol/L; pH 9.8). The reaction mixture was kept at room temperature for 4 h, after which the buffer was changed to TSA buffer (tris-HCl [50 mmol/l] pH 7.75; NaCl [150 mmol/l]; NaN_3 [0.5 g/l]) with NAP-10 and PD-10 columns (GE Healthcare). The mab concentration of the collected eluate was measured with a NanoDrop™ UV-VIS spectrophotometer (Thermo Scientific) at 280 nm.

Diethylenetriaminepentaacetic acid-purified BSA (PerkinElmer Inc.) at 1 g/L was added to mab preparation, after which the solution was filtered through a 0.22 μm pore size filter and stored at 4°C.

The tracer antibodies (7G7, 329 cc, and 1C11 cc) were individually conjugated with 30- to 35-fold molar excess of intrinsically fluorescent [2,2′,2′′,2′′′-{[2-(4-isothiocyanatophenyl) ethylimino]bis-(methylene)bis{4-{[4-(alfa-galactopyranoxy)phenyl]-ethynyl}pyridine-6,2-diyl}bis(methylenenitrilo)}tetra kis(acetato)] europium(III) chelate (Biotechnology, University of Turku) (14). The conjugation reactions were performed in sodium carbonate-bicarbonate buffer at room temperature overnight (18–20 h) and protected from light. The labeled antibody was purified with fast protein liquid chromatography (Pharmacia Ab.) using a Superdex 200 HR 10/30 gel filtration column (Cytiva) and TSA buffer as an eluent. The fractions containing label-conjugated mab were identified with a UV detector and Arcus 1230 fluorometer (Wallac Oy). The selected fractions containing the labeled mab were pooled and 1 g/L of diethylenetriaminepentaacetic acid-purified BSA was added, after which the solution was filtered through a 0.22 μm pore size filter and stored at 4°C.

Long cTnT Assay Protocol

Biotinylated capture antibody was added as 200 ng in 25 μL of Buffer Solution RED (Kaivogen Oy) into streptavidin-coated microtiter wells (Kaivogen) and incubated for 1 h at room temperature. After incubation, the microtiter wells were washed with wash buffer (Kaivogen). The tracer antibodies (100 ng of each) were added to the wells in 40 μL of tracer buffer (Tris-HCl [100 mmol/L] pH 7.75; NaCl [600 mmol/L]; NaN_3 [0.5 g/L]; BSA [25 g/L]; casein [4 g/L] (Calbiochem, Merck Millipore); bovine-γ-globulin [0.6 g/L] (Sigma-Aldrich); native mouse immunoglobulin G [0.8 g/L] (Meridian Life Science Inc.); denaturated mouse immunoglobulin G [0.05 g/L]). Calibrator or sample (30 μL per well) was added to the wells, and the wells were covered with sealing tape and incubated in iEMS incubator/shaker for 1 h at +36°C, 900 rpm (Thermo Electron Corporation). The wells were then washed with the wash buffer and dried in a stream of hot air for 5 minutes. After the plate had cooled down to room temperature, the time-resolved-fluorescence signal was measured with a VictorX4 plate reader (PerkinElmer) with excitation wavelength of 340 nm, emission wavelength of 615 nm, measurement delay of 250 μs, and measurement window of 750 μs.

Total cTnT and Long-to-Total cTnT Ratio

Total cTnT results were produced with the Elecsys hs-cTnT assay (Roche Diagnostics) and Cobas 8000 system 801 module (Roche Diagnostics) by TYKS Laboratories—the FINAS accredited laboratory of Turku University Hospital. The long-to-total cTnT ratio was calculated by dividing our assay result (long cTnT) by the Roche hs-cTnT assay result (total cTnT). The Elecsys Troponin T high-sensitivity assay uses 2 monoclonal antibodies that specifically target the central part of the human cTnT. The capture antibody of the assay recognizes epitope at aar 136–147 and the tracer antibody recognizes the epitope at aar 125–131 (8,12). Thus, the assay targets the middle part of the cTnT molecule detecting all longer and shorter cTnT molecules that contain this part (Fig. 1). The tracer antibody is conjugated with Tris(2,2′-bipyridyl)ruthenium(II)-complex [Ru(bpy)] enabling electrochemiluminescence for signal detection. According to the package insert (08469873500), calibration of the Elecsys Troponin T hs assay is based on the Elecsys Troponin T STAT assay, which is originally standardized against the Enzymun-Test Troponin T method. The reporting limit and the limit of quantitation (LoQ, 10% CV) for the Elecsys hs-cTnT assay were 5 ng/L and 13 ng/L, respectively (15).

Patient Samples

Serum and lithium heparin (LiH) plasma samples were collected from MI patients [ST elevation myocardial infarction (STEMI) n = 66, NSTEMI n = 40] and from healthy volunteers (n = 9) for the TROPOnin FRAGMentation in Myocardial Injury Study (ClinicalTrials.gov Identifier: NCT04465591). The study complies with Declaration of Helsinki as revised in 2013, and the study protocol was approved by the Medical Ethics Committee of the Hospital District of Southwest Finland. The average delay from symptom onset to sampling for the MI patients was 26.8 h (range 3.9–73.1 h). Ninety-seven percent of the STEMI patients and 37% of the NSTEMI patients had undergone revascularization before sampling (time from revascularization to sampling on average 21.5 h, range 2.1–51.5 h, for STEMI and on average 18.0 h, range 0.4–20.8 h, for NSTEMI). All participants provided their written informed consent. Blood samples were collected into 3.5 mL Vacuette CAT Serum Separator Clot Activator (Greiner Bio-One GmbH) and 4 mL Vacutainer LiH (BD Biosciences) tubes. Plasma was centrifuged 15 min at 2200*g* and separated into cryotubes. The same protocol was followed with serum after coagulation. Both serum and plasma were stored at −70°C until analysis. Average time between sample acquisition and centrifugation was 24 min (range 7–61 min). The total processing time prior to freezing the samples was 47 min (range 17–93 min) on average. One STEMI sample had an exceptionally delayed processing time of 215 min. The samples were thawed at room temperature prior to analysis, mixed to ensure homogeneity, and briefly spun using a mini centrifuge to remove any precipitated matrix components.

Long cTnT Assay Evaluation

The limit of blank (LoB) and the limit of detection (LoD) of the long cTnT assay were determined according to the classical approach of Clinical and Laboratory Standards Institute guideline EP17-A2, with the exception of using just one reagent lot (16). For LoB, 5 batches of TSA-BSA buffer as blanks were measured on 5 days as triplicates (n = 75). A nonparametric data analysis approach with risk probability of $\alpha = 0.05$ was used for LoB due to the non-gaussian distribution of data points. For LoD, 6 low-level patient plasma sample pools with long cTnT concentrations ranging from 6.2 to 14.3 ng/L were used. Samples were measured as triplicates on 4 days (n = 72). A parametric analysis approach with β=0.05 was used for LoD due to the gaussian distribution of data points. LoQ was determined as functional sensitivity with an exponential decay function and accuracy goal of 10% CV, adhering to Clinical and Laboratory Standards Institute guideline EP17-A2. In addition, the LoQ with 20% CV was defined. For cTn assays, an accuracy goal of 10% CV for LoQ is commonly used for describing the sensitivity of the assays (15). Concentrations of 10 plasma sample pools containing 5.5 to 44.0 ng/L of long cTnT were measured on 4 days as triplicates (n = 120). Two outliers were excluded from a single pool due to contamination. Dilution linearity was investigated by diluting pooled LiH plasma of 3 (STEMI) patients and in a separate experiment using troponin ITC complex calibrator material in pooled LiH plasma of healthy individuals with serial 2-fold dilutions. Heparin plasma and serum matrices were compared by analyzing LiH plasma and serum samples collected at the same time from 3 STEMI patients.

In Vitro Stability of Long cTnT

Stability of endogenous long cTnT in vitro was investigated by spiking a pool of LiH plasma collected from healthy individuals, or TSA-BSA buffer, with LiH plasma from a STEMI patient (3% of total volume) to a final concentration of 214 ng/L and storing the aliquoted samples at +4°C, +21°C, or +37°C for 24 h or 7 days. The stability was also tested by subjecting separate

aliquots to 1, 3, or 5 freeze–thaw cycles between −20°C and room temperature.

Long, Total, and the Ratio of Long-to-Total cTnT after Symptom Onset

In the TROPOnin FRAGMentation in Myocardial Injury Study, the recorded STEMI (n = 66) and NSTEMI (n = 40) patient data included time from symptom onset to sample acquisition. This patient data was plotted against total cTnT, long cTnT, and the long-to-total cTnT ratio to visualize the relationship after MI.

Statistical Analysis

The distribution of measured values was examined visually and with the Shapiro–Wilk test. Outliers were screened by utilizing the 1.5 times interquartile range rule. The functional sensitivity (LoQ) of the assay was determined by utilizing an exponential decay function in SigmaPlot 16 (Grafiti LLC). Dilution linearity and other linear regression analyses were evaluated in Origin 8 or OriginPro 2024 (OriginLab). For the stability and sample matrix comparison studies, means ± propagated SDs of the recovery values are reported.

RESULTS

We developed a simple immunoassay method that can specifically measure the long cTnT forms efficiently and within adequate concentration range. The clinical evaluation of this assay was previously performed by comparing MI and end-stage renal disease patients in combination with the Elecsys hs-cTnT assay (13).

Analytical Performance of the Assay

The calibration curve of the long cTnT assay produced a linear response ($R^2 = 0.993$; slope = 19.66 ± 0.65; residual sum of squares = 10.5) in the range of 5 to 5000 ng/L (Fig. 2). The determination of LoB and LoD produced results of 4.4 ng/L and 10.8 ng/L, respectively. The assay achieved a LoQ of 30.8 ng/L ($R^2 = 0.986$) with the CV goal of 10%. The assays 20% CV LoQ was found to be at 18.8 ng/L. In sample matrix comparison, the

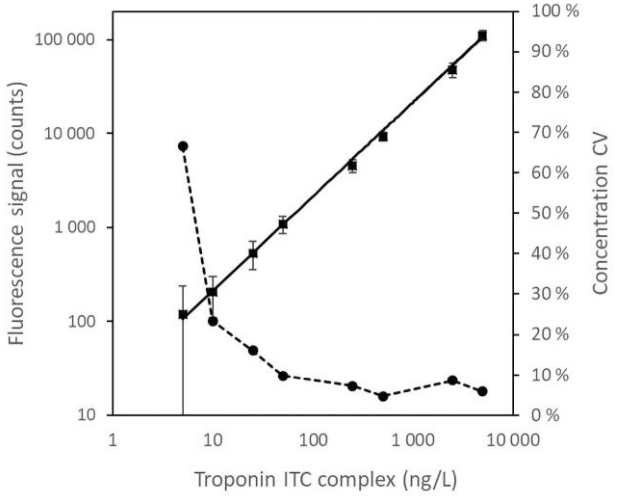

Fig. 2. Calibration curve (solid line) and imprecision profile (dashed line) of the long cTnT assay. The error bars indicate the 95% CI.

serum samples gave significantly lower results than LiH plasma. The long cTnT concentrations of serum samples were 59 ± 6%, 66 ± 10%, and 75 ± 9% of the respective LiH plasma concentrations of selected STEMI patients. To study the linearity, experiments were conducted with troponin ITC-complex and separately with pooled STEMI LiH plasma containing endogenous long cTnT that were step-wise diluted into pooled healthy individual plasma that had been tested to contain no detectable amounts of long cTnT. The assay showed acceptable linearity with endogenous long cTnT ($R^2 = 0.998$; slope = 1.094 ± 0.017; *y*-intercept = −0.43 ± 0.049; residual sum of squares = 21.2) and troponin ITC-complex calibration material ($R^2 = 0.989$; slope = 1.047 ± 0.042; *y*-intercept = −0.18 ± 0.14; residual sum of squares = 132.7) when serially diluted into LiH plasma pool within the range of 20 to 5000 ng.

Stability of Long cTnT

Endogenous long cTnT originating from STEMI LiH plasma and measured with the developed assay was stable in TSA-BSA buffer when stored in a refrigerator (Fig. 3A). Compared to the baseline, yields of 112 ± 10% and 99 ± 12% were obtained at 24 h and 7-day time points, respectively. Incubation at room temperature led to slow decay of the analyte and yields of 88 ± 10% (24 h) and 62 ± 6% (7 days) were obtained. Incubation at +37°C led to a significantly poorer analyte stability with yields of 45 ± 5% (24 h) and 33 ± 3% (7 days).

Endogenous long cTnT from STEMI LiH plasma and spiked into healthy LiH plasma displayed inferior stability at all temperatures compared to TSA-BSA buffer. When the plasma sample was stored in a refrigerator, the analyte showed reasonably slow decay and yields of 82 ± 9% and 67 ± 5% were obtained at 24 h and 7 days, respectively (Fig. 3B). As expected, incubation at higher temperatures resulted in lower stability at both time points. At room temperature, 65 ± 4% and 28 ± 2% were obtained. Almost total analyte

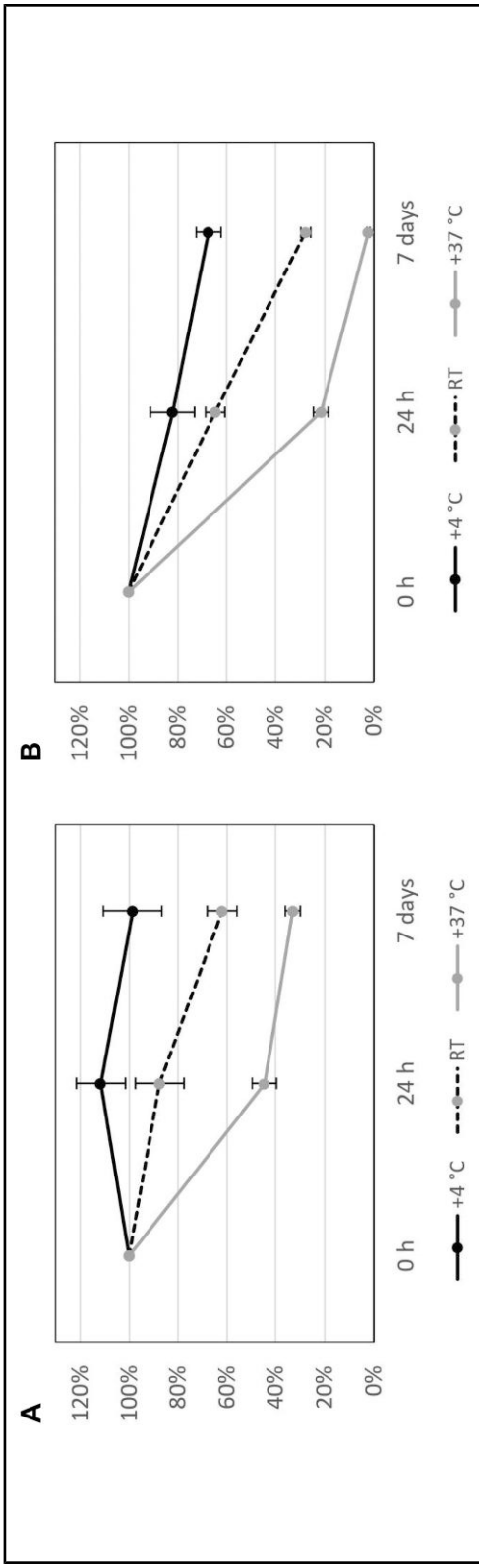

Fig. 3. The stability of long cTnT after 24-hour and 7-day incubation at +4°C, at room temperature, and at +37°C in (A) TSA-BSA buffer and (B) LiH plasma. The error bars indicate the propagated SD.

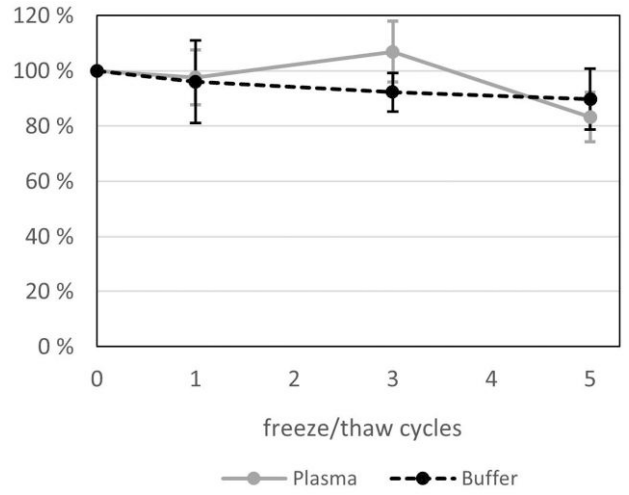

Fig. 4. Analyte stability in comparison to the initial result after 1, 3, and 5 freeze–thaw cycles both in plasma matrix and in TSA-BSA buffer. The error bars indicate the propagated SD.

loss (2 ± 0.5%) was observed after 7-day incubation at +37°C.

Endogenous long cTnT from STEMI LiH plasma showed reasonably good stability following multiple freeze–thaw cycles in both matrices (Fig. 4). After a first freeze–thaw cycle, 98 ± 10% yield was obtained for plasma samples and 96 ± 15% for the sample in TSA-BSA buffer. After 5 complete cycles, the results were 83 ± 9% and 90 ± 11% from the original results, respectively.

Long cTnT Concentration and Long-to-Total cTnT Ratio after Symptom Onset

Long cTnT as an individual parameter showed a clear trend in which higher concentrations predominated early after type 1 MI onset and declined thereafter, whereas total cTnT concentrations were more evenly distributed in time (Table 1, Fig. 5). This observation was confirmed by plotting long cTnT/total cTnT ratios against time after symptom onset (Fig. 5), which also indicated that a higher portion of long cTnT could be observed early after symptom onset and then decreased due to proteolytic degradation of cTnT (Table 1). These observed trends support the notion that long cTnT and long-to-total ratio could be utilized to identify fresh cTnT release.

DISCUSSION

In this article, we report the design and analytical evaluation of an immunoassay designed to detect long forms of cTnT. Previously, we showed in a research letter that long cTnT results obtained with this assay offer superior clinical specificity in differentiating between MI and end-stage renal disease patients than a commercial hs-cTnT assay alone (13).

Growing research interest has focused on the investigation of different forms of cTns and troponin complexes with novel immunoassays (4,17,18). Our assay focuses on the fragmentation at the C-terminal cleavage area of cTnT at aar 189–223 that contains multiple cleavage sites. The capture antibody binds to the C-terminal side and tracer antibodies to the N-terminal side of this area. Our previously published clinical data implies that this selection of epitopes enables the detection of less fragmented cTnT forms that are

Table 1. Long cTnT and troponin ratio in patients with type 1 MI.

Time after symptom onset	n	Long cTnT[a]	Total cTnT[a]	Long/total cTnT ratio[a]
0–24 h	53	226 ng/L [44–972 ng/L]	355 ng/L [115–1480 ng/L]	0.52 [0.27–0.76]
24–48 h	43	293 ng/L [70–627 ng/L]	981 ng/L [333–2370 ng/L]	0.25 [0.16–0.38]
48–74 h	10	155 ng/L [32–552 ng/L]	1820ng/L [237–5960 ng/L]	0.08 [0.06–0.11]

[a]Values reported as median [25th–75th percentile].

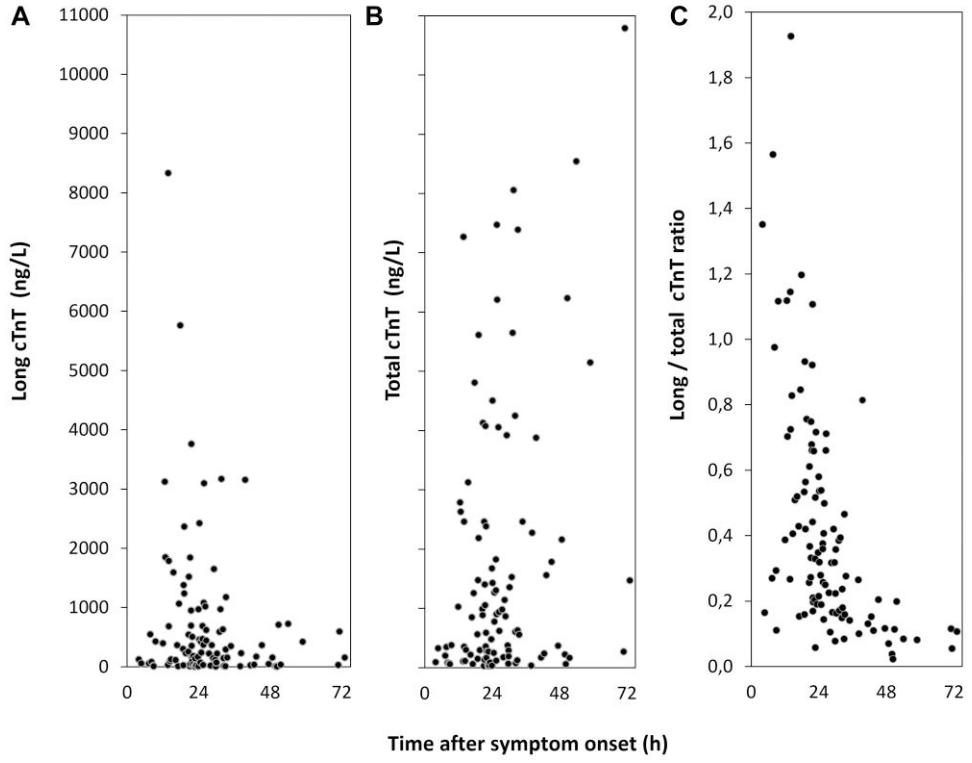

Fig. 5. (A) Long cTnT, (B) total cTnT concentrations, and (C) long-to-total cTnT ratio in samples collected from MI patients at different time points after the onset of symptoms.

more specific to MI compared to the commercial Roche hs-cTnT assays (13). The use of 3 tracer antibodies in long cTnT assay enabled the detection of analyte at an acceptable concentration range with LoD and LoQ (10% CV) values of 10.8 ng/L and 30.8 ng/L, respectively, and thus allowed accurate quantitation and comparison of samples above these levels. The assay produced a linear response in the range of 5 to 5000 ng/L. Due to the assay design, the assay may produce some more signal for the 37 kDa cTnT form (possibility for simultaneous binding of 3 tracer antibodies) than for the 29 kDa

cTnT form (possibility for simultaneous binding of 2 tracer antibodies).

As fragmentation of cTnT is central in our assay, it was necessary to choose a sample matrix that produces minimal in vitro degradation. Our comparative study between serum and LiH plasma matrices is in agreement with previous research: significantly and variably lower concentrations were detected in serum than LiH plasma as thrombin activity in the serum sample contributes to the cTnT degradation (19,20). We further evaluated the stability of long cTnT by spiking samples containing either healthy LiH plasma or our optimized assay buffer with STEMI patient LiH plasma containing endogenous long cTnT and then incubating the samples at different temperatures. Thus, we were able to investigate how the samples should be stored before analysis. The stability of the analyte in LiH plasma at room temperature was mediocre, and this emphasizes that the samples need to be delivered into the laboratory and analyzed as soon as possible or to be frozen after plasma separation in case of extended delays before analysis. More precise evaluation should be conducted to evaluate sufficient timeframes for plasma stability at room temperatures after sampling. Slow analyte decay in the assay buffer at room temperature indicates that a small delay to analysis of a sample does not influence the assay results to any great extent after the sample has been mixed with the assay buffer. The investigated spiked plasma and buffer samples were shown to tolerate freeze–thaw cycles well, which indicates that plasma can be frozen to enhance analyte stability and thus enable longer time between sampling and analysis. Although our study lacks definite kinetic data from individual patients, which is a clear limitation, our data supports the progression of fragmentation at the C-terminal cleavage in time thus leading to decrease of long cTnT and the long to total cTnT ratio as the time passes.

The developed prototype assay provides a clear improvement to previous gel filtration, Western blot, and mass spectrometry-based approaches for investigating cTnT fragmentation in respect to sensitivity, throughput, and simplicity of workflow (5–10). However, for studying samples with low cTnT elevations or low fractions of long cTnT forms, higher sensitivity would be desired.

CONCLUSIONS

Our immunoassay enables sensitive and simple quantitation of intact (37 kDa) and mildly fragmented (29 kDa) forms of cTnT (long cTnT) in LiH plasma. We also addressed the important practical consideration concerning long cTnT stability in LiH plasma and assay buffer. While LiH plasma samples were found to withstand repeated freeze–thaw cycles well, they were susceptible to analyte loss after prolonged storage at room temperature or higher temperatures. We also showed a trend of decreasing concentration and fraction of long cTnT in type 1 MI patients with the passing of time after the symptom onset.

Data Availability: Data will be made available on reasonable request from the corresponding author.

Nonstandard Abbreviations: cTn, cardiac troponin; cTnT, cardiac troponin T; MI, myocardial infarction; hs-cTn, high-sensitivity cardiac troponin; NSTEMI, non-ST elevation myocardial infarction; aar, amino acid residue; mab, monoclonal antibody; TSA, Tris-buffered saline with azide; BSA, bovine serum albumin; LoQ, limit of quantitation; LiH, lithium heparin; STEMI, ST elevation myocardial infarction; LoB, limit of blank; LoD, limit of detection.

Author Contributions: *The corresponding author takes full responsibility that all authors on this publication have met the following required criteria of eligibility for authorship: (a) significant contributions to the conception and design, acquisition of data, or analysis and interpretation of data; (b) drafting or revising the article for intellectual content; (c) final approval of the published article; and (d) agreement to be accountable for all aspects of the article thus ensuring that questions related to the accuracy or integrity of any part of the article are appropriately investigated and resolved. Nobody who qualifies for authorship has been omitted from the list.*

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