Molecular Heterogeneity Has a Major Impact on the Measurement of Circulating N-Terminal Fragments of A- and B-Type Natriuretic Peptides

MINNA ALA-KOPSALA,1 JARKKO MAGGA,3 KEIJO PEUKURINEN,3 JAANA LEIPÄLÄ,4 HEIKKI RUSKOAHO,2 JUHANI LEPPÄLUOTO,1 and OLlli VUOLTEENAH01*

Background: The N-terminal fragments of A- and B-type natriuretic peptides (NT-proANP and NT-proBNP) are powerful markers of cardiac function. The current assays require refinement with regard to standardization with native calibrators and the ability to detect the actual circulating forms.

Methods: The following peptides were prepared with recombinant methods: NT-proANP, NT-proBNP, proBNP1–108, and Tyr0-proBNP77–108. Fifteen peptides of 13–22 amino acids, spanning the sequences of NT-proANP and NT-proBNP, were prepared by solid-phase peptide synthesis. Two immunoassays for NT-proANP and four for NT-proBNP were set up, each with a different epitope specificity. The assays were applied for the measurement of NT-proANP and NT-proBNP in healthy individuals and in patients with acute myocardial infarction. The circulating molecular forms were analyzed by gel-filtration and reversed-phase HPLC.

Results: According to the HPLC analyses, circulating NT-proANP consists mainly of the full-length peptide, with some degradation at both ends. In contrast, circulating NT-proBNP is very heterogeneous. Most immunoreactive NT-proBNP is significantly smaller in size than NT-proBNP1–76, with truncation at both termini. The smallest fragments can be detected by assays directed at the central part of NT-proBNP only; assays directed at the ends gave 30–40% lower values. Despite the difference, the various assays correlated reasonably well with each other (r² = 0.77–0.85). In patients with acute myocardial infarction, NT-proANP and NT-proBNP concentrations were 1.8–2.3 and 4.2–4.5 times higher than in healthy individuals. The development of heart failure further increased the concentrations.

Conclusions: Molecular heterogeneity of the circulating forms causes a serious risk of preanalytical errors in assays for NT-proBNP and, to a lesser extent, NT-proANP. The development of a sandwich assay for NT-proBNP would be especially challenging. The most robust and reliable assays use antibodies directed at the central portions of NT-proANP or NT-proBNP.

Background: Natriuretic peptides are cardiac hormones regulating salt and fluid homeostasis. They reduce cardiac load by causing natriuresis and diuresis, relaxing vascular smooth muscle, and inhibiting the renin-angiotensin-aldosterone system (1). The prohormones of A- and B-type natriuretic peptides are produced by cardiac myocytes and are cleaved on secretion into the biologically active peptides (ANP and BNP) and their N-terminal propeptides (NT-proANP and NT-proBNP). The synthesis of proANP and proBNP is increased during mechanical load and neurohumoral stimulation of the heart, and ANP and BNP as well as their N-terminal propeptides are released into the circulation (2). Cardiac impairment is associated with increased concentrations of all four peptides. Measurement of natriuretic peptides has been shown to be useful in predicting the risk of cardiovascular events and mortality in a variety of settings.

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5 Nonstandard abbreviations: ANP and BNP, A- and B-type natriuretic peptide, respectively; NT-proANP and NT-proBNP, N-terminal profragments of ANP and BNP, respectively; Tyr-BNP, Tyr0-proBNP77–108; GF-HPLC, gel-filtration HPLC; RP-HPLC, reversed-phase HPLC; AMI, acute myocardial infarction; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; GST, glutathione S-transferase; and EC50, median effective concentration.
in the diagnosis of heart failure (3–5). High concentrations correlate with poor prognosis in myocardial infarction or heart failure (6, 7). Moreover, plasma concentrations of natriuretic hormones are useful in guiding therapy in heart failure patients (8–10). NT-proANP and NT-proBNP are more stable and have longer half-lives in the circulation than do ANP and BNP. Thus, the N-terminal peptides are potentially better markers of prolonged cardiac overload than are the biologically active peptides (2).

Both competitive and sandwich assays have been developed for measuring circulating NT-proANP and NT-proBNP; these assays use radioactive, enzymatic, or chemiluminescence labels (11–22). Measured concentrations vary greatly between different assays, especially in the case of NT-proBNP (23, 24). Furthermore, there is some debate about the actual circulating forms. NT-proANP has been reported to circulate in humans almost exclusively as intact NT-proANP1–98 (25). Alternatively, proANP1–108 has been found to be broken down into NT-proANP1–30, NT-proANP31–67, and NT-proANP68–108, which have vasoactive properties (26). Chromatographic studies of proBNP have indicated the presence in human circulation of high-molecular-weight proBNP together with a shorter NT-proBNP peptide (27, 28).

The aims of the present study were to clarify the nature of immunoreactive NT-proANP and NT-proBNP in human blood and to use this information to set up immunoassays, calibrated with full-length peptides, capable of measuring the actual circulating forms of the peptides. We report here the production and purification of recombinant human NT-proANP, NT-proBNP, proBNP1–108, and Tyr0-proBNP77–108 (Tyr-BNP) as well as the development of six separate competitive immunoassays for NT-proANP and NT-proBNP. Each assay uses a recombinant full-length calibrator but has a different epitope specificity. The circulating forms of immunoreactive NT-proANP and NT-proBNP were characterized by gel-filtration and reversed-phase HPLC (GF-HPLC and RP-HPLC, respectively). Furthermore, five of the assays were used to measure peptide concentrations in healthy persons and in patients with acute myocardial infarction (AMI).

**Materials and Methods**

**Production and Purification of Glutathione S-Transferase Fusion Proteins**

The expression constructs were made by subcloning reverse transcription-PCR fragments of the ANP or BNP genes into pGEX-4T-1 vector (Amersham Pharmacia Biotech). The constructs were designed to contain the sequence of the human ANP gene coding for amino acids 1–98 of the peptide or the sequences of the human BNP gene coding for amino acids 1–76, 1–108, or 77–108 of the peptide. The cDNA first strands were synthesized from human atrial RNA with use of sequence-specific 3′ primers including the termination codon (TGA) and an EcoRI linker (NT-proANP, 3′-GCGAATTTCACCGAGGGGC-AGTGAGC-5′; NT-proBNP, 3′-GCGAATTTCCTCATCGTGTGCCCCGCAG-5′; proBNP and Tyr-BNP, 3′-GCGAATTTCATTAATGCCGCCTAGCACA-5′). Subsequent PCR amplifications were done using the same 3′ primers and 5′ primers that included BamHI linkers (NT-proANP, 5′-GCGGATCAATCCCATGTAACATGCCG-3′; NT-proBNP and proBNP, 5′-GCGGATCCACCCGGCTGGCGCACCCG-3′). The 5′ primer for Tyr-BNP contained a BamHI linker and a codon for tyrosine (5′-GCGGATCCTACAGCACCAGATGGTGCAA-3′). The PCR fragments were subcloned into the EcoRI/BamHI site of pGEX-4T-1 vector. The nucleotide sequences and reading frames of the constructs were confirmed by automated sequencing on an ABI Prism 310 Genetic Analyzer (Applied Biosystems).

An overnight culture of *Escherichia coli* BL21 transformed with recombinant plasmid was diluted 1:100 in 2× YTA (yeast extract, tryptone, NaN, pH 7) and grown at 37°C until the absorbance at 600 nm reached 0.6. Isopropyl-1-thio-β-D-galactopyranoside was added to a final concentration of 0.1 mmol/L, and the culture was incubated for an additional 1–2 h. The bacterial cells were harvested by centrifugation at 7000g for 10 min at 4°C, resuspended in phosphate-buffered saline (50 μL for 1 mL of culture), and sonicated. The cell lysate was cleared by centrifugation at 7000g for 15 min. The supernatant was applied to a glutathione-Sepharose 4B column and washed three times with phosphate-buffered saline. The fusion proteins were eluted with 10 mmol/L glutathione in 50 mmol/L Tris-HCl, pH 8.0, and stored in aliquots at −20°C. The products were analyzed by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

**Preparation and Purification of Recombinant NT-proANP, NT-proBNP, proBNP1–108, and Tyr0-proBNP77–108**

Recombinant peptides were released from the glutathione S-transferase (GST) fusion partner by treating with thrombin at room temperature for 1 h (1 U/100 g of protein). The peptides were purified by RP-HPLC on a Vyduc C4 HPLC column [25 × 1 cm (i.d.); The Separations Group]. The column was eluted with a linear 40-min gradient from 20–50% acetonitrile in aqueous 1 mL/L trifluoroacetic acid. The elution rate was 2 mL/min, and the absorbance at 220 nm was measured to monitor the purity of products. The peak fractions were collected manually. The peptides were quantified based on absorbance at 214 nm by GF-HPLC calibrated with bovine serum albumin. GF-HPLC was performed on a Waters ProteinPak-125 [300 × 3.9 mm (i.d.)] column eluted with 400 mL/L acetonitrile containing 1 mL/L trifluoroacetic acid at 1 mL/min. The peptides were also separated by 12% SDS-PAGE gel using Tris-tricine electrophoresis buffer. The N-terminal sequences of the peptides were confirmed by 10 cycles of automated Edman degradation in an ABI 477A gas phase sequencer (Applied Biosystems). A QToF
I mass spectrometer (Micromass Ltd.) was used to determine the molecular masses of recombinant NT-proANP and NT-proBNP.

PEPTIDE SYNTHESIS

Synthetic peptides (NT-proANP1-20, NT-proANP20-38, NT-proANP35-46, NT-proANP39-59, NT-proANP40-60, NT-proANP70-80, NT-proANP80-90, NT-proANP90-108, NT-proBNP1-22, NT-proBNP16-29, NT-proBNP16-29, NT-proBNP22-40, NT-proBNP41-56, Tyrα-NT-proBNP57-76, and Tyrα-NT-proBNP57-76) were assembled using Fmoc chemistry with an ABI 433A Peptide Synthesizer (Applied Biosystems).

ANTISERA

The affinity-purified GST/NT-proANP fusion protein was used as such as an immunogen. Before immunization, the peptide immunogens NT-proANP1-20, NT-proANP16-29, NT-proBNP1-22, NT-proBNP16-29, NT-proBNP22-40, NT-proBNP41-56, Tyrα-NT-proBNP57-76, and Tyrα-NT-proBNP57-76 were coupled by water-soluble carbodiimide to bovine thyroglobulin or horseshoe crab hemocyanin. Goats received injections at multiple sites in the back (1.0 mg of immunogen emulsified in an equal volume of Freund’s complete adjuvant; Difco Laboratories). Boosters of 0.5 mg in Freund’s incomplete adjuvant were given two to four times at 2- to 3-week intervals, and the goats were bled 14 days after the injections. The cross-reactivities of the antisera were measured by assaying serial dilutions (0.001–1 μmol/L) of the following vasoactive peptides: human proANP99-126 (ANP1-29), rat proANP99-126 (ANP1-29), human proBNP77-108 (BNP1-32), human C-type natriuretic peptide, rat adrenomedullin, human endothelin-1, human proBNP1-108, human NT-proBNP, and human NT-proANP. The epitope specificities of the antisera were tested using the synthetic peptides described above.

TRACER PREPARATION

Recombinant NT-proANP and NT-proBNP (1.5 μg) were radioiodinated with 0.5 mCi of Na125I in the presence of 5 μg of chloramine-T in 0.25 mol/L phosphate buffer (pH 7.5) for 15 s, followed by the addition of 10 μg of sodium disulfite. The reaction mixture was purified by gel-filtration chromatography with Sephadex G-25 (Amersham Pharmacia Biotech) followed by RP-HPLC in a Symmetry C18 column [150 × 4.6 mm (i.d.); Waters]. The peptides were eluted with a 30-min gradient of 20–50% acetonitrile in aqueous 1 mL/L trifluoroacetic acid at a flow rate of 1 mL/min. Fractions of 1 mL were collected and monitored for radioactivity in a MultiGamma counter (Wallac). The iodinated peptides were usable for at least 5 weeks when stored in aliquots at −20 °C.

ASSAY PROCEDURES

RIAs for NT-proANP1-20 and NT-proANP46-79. Recombinant NT-proANP was used as a calibrator and a tracer in both assays. The following assay buffer was used for all dilutions: 0.04 mol/L sodium hydrogen phosphate, 0.01 mol/L sodium dihydrogen phosphate, 0.1 mol/L NaCl, 1 g/L gelatin, 0.5 mL/L Triton X-100, pH 7.4. Calibrators (60–6000 pmol/L) or plasma or serum samples were pipetted in duplicates of 25 μL and incubated for 16–24 h at 4 °C with 100 μL of antiserum solution (1:20 000 dilution for antiserum to NT-proANP46-79 and 1:4 000 dilution for antiserum to NT-proANP1-20) and 100 μL of tracer solution containing ~8000 cpm of the appropriate radioiodinated peptide. Bound and free fractions were separated by precipitation with donkey anti-goat IgG (Scantibodies Laboratory, Inc., and Linco Research, Inc.) in 0.5 mL of 80 g/L polyethylene glycol 6000 containing normal goat serum as a carrier. After centrifugation, the pellet was counted for radioactivity (Clinigamma 1272; Wallac).

RIAs for NT-proBNP1-22, NT-proBNP10-29, and NT-proBNP57-76. Recombinant NT-proBNP was used as the calibrator and tracer in all assays. Calibrators (40–4000 pmol/L) or plasma or serum samples were pipetted in duplicates of 25 μL and incubated for 16–24 h at 4 °C with 100 μL of antiserum solution (final dilutions, 1:10 000 for antiserum to NT-proBNP1-22 and 1:50 000 for antisera to NT-proBNP10-29 and NT-proBNP57-76) and 100 μL of tracer solution containing ~8000 cpm of radioiodinated peptide. Bound and free fractions were separated with double-antibody precipitation as described above. To be able to measure the low NT-proBNP concentrations in healthy individuals, we used an alternative incubation procedure with increased sensitivity. The tracer was added after incubation for 16–24 h, and the calibrators covered the range 16–1600 pmol/L. Otherwise, the procedure was identical to that described above.

RIAs for NT-proBNP5-24. Calibrators (62.5–1000 pmol/L recombinant NT-proBNP) or plasma or serum samples were pipetted in duplicates of 50 μL and incubated for 16–24 h at 4 °C with 100 μL of antiserum solution at a dilution 1:14 000. Tracer solution (100 μL) containing ~8000 cpm of iodinated peptide was then added, and the incubation was continued for an additional 16–24 h. Bound and free fractions were separated with double-antibody precipitation as described above.

RIA VALIDATION

Serial dilutions of human plasma and serum were assayed against the NT-proANP and NT-proBNP calibrators to test for parallelism. Assay linearity was analyzed by linear regression analysis with normal human plasma to which the recombinant NT-proANP (100–5000 pmol/L) or NT-proBNP (50–3200 nmol/L; n = 4 each) had been added. The within- and between-assay precision was assessed by measuring 10 replicates of plasma or serum pools with low, medium, and high concentrations of the peptides. The functional sensitivities were analyzed with normal plasma and serum samples as day-to-day CVs <20% (5 replicates on 5 different days). Recoveries
were determined by supplementing the normal plasma pool with 500, 2000, or 5000 pmol/L NT-proANP or 200, 800, or 3200 pmol/L NT-proBNP (n = 8 for each).

OTHER RIAs
NT-proANP$_{99-96}$ was measured according to the published RIA method (15). For measurement of the biologically active BNP (proBNP$_{77-108}$), the plasma and serum samples were first extracted with Sep-Pak C$_{18}$ cartridges (Waters). Calibrators [1–100 pmol/L of synthetic human BNP (proBNP$_{77-108}$) purchased from Bachem] or plasma or serum extracts were pipetted in duplicates of 200 μL and incubated for 16–24 h at 4°C with 100 μL of rabbit antiserum against human proBNP$_{77-108}$ at a dilution 1:30 000. Radioiodinated recombinant Tyr$_0$proBNP$_{77-108}$ (~8000 cpm) was added, and the incubation was continued for an additional 16–24 h. The bound and free fractions were separated as described above, with the exception that goat anti-rabbit IgG and normal rabbit serum were used.

RP-HPLC AND GF-HPLC ANALYSIS OF HUMAN PLASMA AND SERUM
Each human plasma or serum sample (300 μL) was mixed with 200 μL of 1 mL/L trifluoroacetic acid in acetonitrile and cleared by centrifugation at 10 000 g for 10 min. The supernatant was passed through a 0.45 μm Millipore filter, and the filtrate was subjected to RP- or GF-HPLC analysis. For RP-HPLC, the sample was applied to a Vydac C$_4$ [150 × 4.6 mm (i.d.); Waters] and eluted with 40% acetonitrile in aqueous 1 mL/L trifluoroacetic acid. The elution rate was 1 mL/min, and 1-mL fractions were collected. For GF-HPLC analysis, the sample was applied to a Protein-Pak 125 GF-HPLC column [300 × 3.9 mm (i.d.); Waters] and eluted with 40% acetonitrile in aqueous 1 mL/L trifluoroacetic acid. The flow rate was 1 mL/min, and 0.5-mL fractions were collected. Fractions were dried in a SpeedVac concentrator and dissolved in 0.5 mL of RIA assay buffer for use in the RIAs.

SAMPLE COLLECTION
Venous blood samples (serum) from 100 healthy individuals (blood donors; age range, 20–65 years; 50 females and 50 males) were collected into Vacutainer Tubes. Blood samples (EDTA plasma) were also obtained from 86 patients with AMI (age range, 39–108 years; 19 females and 65 males) (29) at entry to the emergency unit, at 96 h, and/or at discharge from the hospital, so that the total sample count was 206. A few serum samples were also obtained for use in the HPLC studies. The characteristics of study participants are presented in Table 1. All patients showed ST-segment elevation on electrocardiograms and were treated with the thrombolytic therapy. One week after admission to hospital, the ejection fraction was ≤40% in 17 patients as determined by echocardiography. In addition, venous blood samples (serum) from five premature infants were used for the initial characterization of the circulating forms of NT-proANP and NT-proBNP because of their extremely high peptide content. Plasma and serum samples were stored at −20°C until analysis.

Informed consent was obtained from all volunteers and patients (or their parents). The investigation conformed with the principles outlined in the Declaration of Helsinki and was approved by the ethics committees of the Universities of Kuopio and Helsinki.

STATISTICS
Our results are reported as the mean (SE). Concentrations below the detection limits are reported as the value for the lowest calibrator. Correlation coefficients were calculated by linear regression analysis. Comparison between two groups was performed using unpaired nonparametric analysis (Mann–Whitney). Statistical significance was defined as $P < 0.05$.

Results

| Table 1. Characteristics of the 86 patients with AMI. |
|-----------------|--------|
| **Characteristic** | **Value** |
| Mean (SD) age, years | 61 (11) |
| Male sex, no. of patients (%) | 66 (77) |
| AMI, no. of patients (%) | 86 (100) |
| Q-wave | 52 (62) |
| Thrombolytic treatment | 86 (100) |
| Reperfusion | 49 (58) |
| Complications (hemorrhage) | 8 (9.5) |
| Arrhythmia | 13 (15) |
| Heart failure | 24 (29) |
| Medications on admission, no. of patients (%) | |
| β-Blockers | 19 (22) |
| Nitrate | 12 (14) |
| Aspirin | 12 (14) |
| ACE$^a$ inhibitor | 11 (13) |
| CCB | 12 (14) |
| Diuretics | 4 (5) |
| Digitalis | 1 (1) |
| Hypolipidemic agents | 7 (8) |

$^a$ ACE, angiotensin-converting enzyme; CCB, calcium channel blocker.
the fusion proteins appeared as single bands without any major contaminants, and the sizes of the fusion proteins were as expected (Fig. 1A; ~40, ~37.5, ~41, and ~32.5 kDa, respectively).

Recombinant human NT-proANP, NT-proBNP, proBNP1–108, and Tyr0-proBNP77–108 were released from their fusion partners by digestion with thrombin (31). An extra Gly-Ser dipeptide, originating from the cloning vector, was left at the amino terminus of the recombinant peptides. The peptides were further purified from the GST partner, thrombin, and other contaminants by RP-HPLC (Fig. 1, B and C) with a yield of 0.1–0.5 mg per liter of original bacterial culture. The sizes of the peptides were ~11 kDa for NT-proANP, ~8.6 kDa for NT-proBNP, ~12 kDa for proBNP1–108, and ~3.5 kDa for Tyr0-proBNP77–108 as detected by SDS-PAGE and GF-HPLC, corresponding to those expected from the amino acid sequences.

The molecular masses of recombinant NT-proANP and NT-proBNP were further ascertained with a mass spectrometer. The obtained masses were 10 767 (8.16) Da for NT-proANP and 8279 (0.43) and 8603 (0.4) Da for NT-proBNP. The identities were also confirmed by N-terminal sequencing, which yielded the unambiguous sequences GSNPMYNAVS for NT-proANP (Fig. 1B), GSHPLGSPGS for NT-proBNP (Fig. 1C) and proBNP1–108, and GSYSPKMVQG for Tyr0-proBNP57–76 (data not shown), consistent with the vector-derived N-terminal Gly-Ser dipeptide followed by the native peptide sequences. The characterized material from a single HPLC purification of NT-proANP or NT-proBNP was used for assay development. These two recombinant peptides are referred to as NT-proANP and NT-proBNP in the text.

**Production of Antisera and Development of Immunoassays**

GST fusion proteins were used as such as immunogens. In addition, we immunized goats with synthetic peptide fragments of NT-proANP and NT-proBNP (NT-proANP1–20, NT-proANP1–22, NT-proBNP1–108, NT-proBNP1–108, NT-proBNP1–108, and Tyr0–NT-proBNP77–108) coupled to thyroglobulin or hemocyanin. After boosters (2, 3), test bleeds showed 40% binding of the appropriate tracers at final dilutions 1:10 000 to 1:50 000 (Table 2). However, antiserum raised against NT-proBNP1–108 bound to NT-proBNP with a very low titer. The epitope of the GST-NT-proANP antiserum could not be assigned to a simple short sequence, but instead located broadly in the region of amino acids 46–79. Thus, it is referred to as antiserum NT-proANP46–79. The epitopes of antisera NT-proANP1–22, NT-proBNP1–22, NT-proBNP10–29, and NT-proBNP57–76 were also tested and were sited in the regions of amino acids 5–20, 15–25, and 57–70, respectively.

Recombinant NT-proANP and NT-proBNP were radiiodinated at their tyrosine residues and used as tracers in the RIAs. The recombinant peptides were used as calibrators. Typical calibration curves for the NT-proANP46–79 and NT-proBNP10–29 assays together with
proANP46 pmol/L (1236 pmol/L, and that for the NT-proANP/H11005 NT-proBNP concentrations of NT-proANP or NT-proBNP (n assays (NT-proBNP1 ANP (proANP99 antisera relative to other vasoactive peptides human [ANP (proANP99–126), rat ANP (proANP99–126), human BNP (proBNP77–106), C-type natriuretic peptide, rat adrenomedullin, and human endothelin-1] were <0.03%. The NT-proANP antisera did not recognize recombinant human NT-proBNP, and none of the NT-proBNP antisera recognized recombinant human NT-proANP (<0.03%)..

The NT-proANP assays (NT-proANP1–20 and NT-proANP46–79) were highly linear in the range 100–5000 pmol/L (r² = 0.999). The NT-proBNP assays (NT-proBNP1–22, NT-proBNP10–29, and NT-proBNP57–76) were linear at 100–3200 pmol/L (r² = 0.9996, 0.994, and 0.999, respectively), and the NT-proBNP5,24 assay was linear at 100–800 pmol/L (r² = 0.995). The detection limits varied between 16 and 63 pmol/L (Table 2). The functional sensitivities for NT-proANP and NT-proBNP were 100 and 70–80 pmol/L, respectively. The median effective concentration (EC₅₀) for the NT-proANP1–20 assay was 1236 pmol/L, and that for the NT-proANP46–79 assay was 389 pmol/L. The EC₅₀ values for the different NT-proBNP assays (NT-proBNP1–22, NT-proBNP10–29, and NT-proBNP57–76) were 383, 325, 576, and 520 pmol/L, respectively. The within- and between-assay imprecisions (CVs) were always <15% and <20%, respectively, and usually were markedly better (Table 2). The recoveries of the analytes varied between 58% and 104%. The analytes were recovered equally well from plasma and serum. The assay characteristics are summarized in Table 2.

### Table 2. Characteristics of NT-proANP and NT-proBNP RIAs.

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<th>Antiserum titera</th>
<th>Detection limit, b pmol/L</th>
<th>EC₅₀, c pmol/L</th>
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- **a** Titer was determined as the antiserum dilution that bound 40% of the radioiodinated tracer.
- **b** Detection limit was calculated as pmol/L at 95% displacement of the radioiodinated tracer.
- **c** Detection limit with delayed incubation.
- **d** Within- and between-assay imprecision was assessed by measuring pooled plasma samples with low (295–466 pmol/L for NT-proANP, 131–297 pmol/L for NT-proBNP, middle (1714–2890 pmol/L for NT-proANP, 300–474 pmol/L for NT-proBNP), and high (3076–4559 pmol/L for NT-proANP, 1924–2549 pmol/L for NT-proBNP) concentrations of NT-proANP or NT-proBNP (n = 10).
- **e** Recoveries were determined by supplementing the normal plasma pool with 500, 2000, or 5000 pmol/L NT-proANP or 200, 800, or 3200 pmol/L NT-proBNP; n = 8 each.
- **f** Concentration was above the highest calibrator of the assay.

HPLC analyses of blood samples

HPLC analyses were performed to characterize the molecular forms of the immunoreactive NT-proANP and NT-proBNP present in human circulation (Figs. 3 and 4). The HPLC fractions were analyzed with antisera that had epitope specificities in different parts of the peptides. A single fairly broad area of immunoreactivity was detected with all of the NT-proANP assays (NT-proANP1–20, NT-proANP46–79, and NT-proANP79–98) in the RP-HPLC (Fig. 3A) and GF-HPLC (Fig. 4A) analyses of human plasma, peaking at the elution position of the recombinant NT-proANP calibrator. The NT-proANP1–20 and NT-proANP79–98 antisera, however, consistently detected 30–50% lower concentrations of immunoreactive NT-proANP in plasma samples compared with the NT-proANP46–79 antiserum (Table 3). The difference was even greater (30–70%) with serum. Furthermore, the NT-proANP46–79 antiserum detected immunoreactivity in the serum samples eluting after the main peak in GF-HPLC and thus having lower molecular masses. These results indicate that there is some structural heterogeneity in circulating immunoreactive NT-proANP as a result of N- and C-terminal truncation, which can damage the epitopes of terminal antisera.

The major peak of immunoreactive NT-proBNP in RP-
HPLC analysis of human plasma was detected by all three NT-proBNP assays (NT-proBNP1–22, NT-proBNP10–29, and NT-proBNP57–76; Fig. 3C). The broad peak eluted before the NT-proBNP calibrator, showing that the immunoreactivity was attributable to material that was less hydrophobic, and thus probably smaller in size, than NT-proBNP1–76. RP-HPLC analysis of serum revealed the presence of even less hydrophobic immunoreactivity, which was recognized with the NT-proBNP10–29 antisera only (Fig. 3D).

The results from the plasma GF-HPLC analyses were consistent with the RP-HPLC data. NT-proBNP immunoreactivity eluted as a broad region peaking later than the NT-proBNP calibrator, indicating a molecular mass of ~6–8 kDa (Fig. 4C). The GF-HPLC analysis of immunoreactive NT-proBNP in human serum revealed an even broader peak and indicated the presence of immunoreactive material with a molecular mass as low as 3.5 kDa (Fig. 4D). Thus, our HPLC data show that circulating immunoreactive NT-proBNP is strongly heterogeneous. NT-proBNP appears to fragment extensively from both ends, and the smallest components can be detected only with antisera directed at the central region of NT-proBNP.

NT-proANP, NT-proBNP, and BNP in human plasma or serum

Serum samples from healthy individuals of both sexes (age range, 20–65 years) were measured with the different NT-proANP and NT-proBNP immunoassays (Fig. 5). The mean (SD) concentrations in healthy persons measured with assays specific to NT-proANP46–79 and NT-proANP46–79 were 141 (54) and 255 (95) pmol/L (n = 100), respectively. The corresponding 95% confidence intervals were 130–151 and 236–274 pmol/L. The mean (SD) concentrations in healthy individuals measured with assays specific to NT-proBNP5–24, NT-proBNP10–29, and NT-proBNP57–76 were 86 (41) pmol/L (21 pmol/L below the detection limit; n = 100), 86 (32) pmol/L (all detectable with the assay with delayed addition of tracer), and 59 (37) pmol/L (6 pmol/L below the detection limit for the assay with delayed addition of tracer), respectively. The corresponding 95% confidence intervals were 78–94, 79–92, and 51–66 pmol/L.

We used the five new assays, together with an extracted in-house BNP (proBNP 77–108) assay for reference, to measure natriuretic peptide concentrations in 206 plasma samples obtained from patients after AMI (Table 3 and Fig. 5). All N-terminal assays revealed significantly increased concentrations in the AMI patients at 96 h (n = 45) compared with healthy individuals (P < 0.001). The mean (SD) concentrations of NT-proANP1–20 and NT-proANP 46–79 were increased 2.3-fold [141 (5) vs 326 (40) pmol/L]/1.8-fold [255 (10) vs 467 (38) pmol/L], respectively. The increases in NT-proBNP5–24, NT-proBNP10–29, and NT-proBNP57–76 were more pronounced: 4.2-fold [86 (3) vs 364 (41) pmol/L], 4.5-fold [86 (3) vs 391 (61) pmol/L], and 4.5-fold [59 (4) vs 268 (48) pmol/L], respectively. The development of heart failure (ejection fraction ≤40%) was associated with an additional 1.4- to 1.7-fold increase in NT-proANP1–20 and NT-proANP46–79 concentrations [326 (40) vs 552 (121) pmol/L and 467 (38) vs 658 (88) pmol/L], respectively; n = 16; P < 0.05]. The corresponding heart failure-associated increases for NT-proBNP5–24, NT-proBNP10–29, and NT-proBNP57–76 were 1.6- to 1.7-fold [364 (41) vs 570 (63), 391 (61) vs 630 (107), and 268 (48) vs 463 (75) pmol/L], respectively; P < 0.01]. BNP (proBNP 77–108) concentrations were measured in 50 patients with AMI at 96 h. The mean BNP concentrations in the absence and presence of heart failure were 33 (7) pmol/L (n = 40) and 61 (14) pmol/L (n = 10), respectively (P = 0.011).

The results obtained with the NT-proANP1–20 and
Fig. 3. RP-HPLC analysis of plasma and serum samples from patients with AMI.

RP-HPLC fractions of plasma (A) and serum (B) samples were assayed for NT-proANP (●, NT-proANP1-20; ○, NT-proANP16-70; ▲, NT-proANP79-98). The fractions in plasma (C) and serum (D) were assayed for NT-proBNP (●, NT-proBNP1-22; ○, NT-proBNP10-29; ▲, NT-proBNP57-76). Recombinant NT-proANP, NT-proBNP, and proBNP1-108 and purified rat proANP were used to calibrate the column.

Fig. 4. GF-HPLC analysis of plasma and serum samples from patients with AMI.

Immunoreactive GF-HPLC fractions of plasma (A) and serum (B) samples were assayed for NT-proANP (●, NT-proANP1-20; ○, NT-proANP16-70; ▲, NT-proANP79-98). The fractions in plasma (C) and serum (D) were assayed for NT-proBNP (●, NT-proBNP1-22; ○, NT-proBNP10-29; ▲, NT-proBNP57-76). Recombinant NT-proANP, NT-proBNP, and proBNP1-108 and purified rat proANP, bovine serum albumin (V0), and 125I-H11002 (V∞) were used to calibrate the column.
Table 3. Ratios (%) between different NT-proANP and NT-proBNP concentrations and NT-proANP<sub>46–79</sub> and NT-proBNP<sub>10–29</sub> concentrations in normal serum, in plasma from AMI patients, and in HPLC fractions of plasma and serum.

<table>
<thead>
<tr>
<th>Source</th>
<th>NT-proANP&lt;sub&gt;46–79&lt;/sub&gt;/NT-proANP&lt;sub&gt;1–20&lt;/sub&gt;</th>
<th>NT-proANP&lt;sub&gt;70–98&lt;/sub&gt;/NT-proANP&lt;sub&gt;46–79&lt;/sub&gt;</th>
<th>NT-proBNP&lt;sub&gt;1–22&lt;/sub&gt;/NT-proBNP&lt;sub&gt;10–29&lt;/sub&gt;</th>
<th>NT-proANP&lt;sub&gt;46–79&lt;/sub&gt;/NT-proANP&lt;sub&gt;1–20&lt;/sub&gt;</th>
<th>NT-proBNP&lt;sub&gt;10–29&lt;/sub&gt;/NT-proBNP&lt;sub&gt;1–20&lt;/sub&gt;</th>
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<td>AMI plasma&lt;sup&gt;a&lt;/sup&gt;</td>
<td>65 (52–78)</td>
<td>60 (60–70)</td>
<td>54 (53–61)</td>
<td>105 (87–135)</td>
<td>68 (54–82)</td>
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<tr>
<td>RP-HPLC of plasma&lt;sup&gt;b&lt;/sup&gt;</td>
<td>58 (56–65)</td>
<td>60 (60–70)</td>
<td>54 (53–61)</td>
<td>105 (87–135)</td>
<td>68 (54–82)</td>
</tr>
<tr>
<td>GF-HPLC of plasma&lt;sup&gt;b&lt;/sup&gt;</td>
<td>50 (46–54)</td>
<td>65 (62–70)</td>
<td>57 (51–59)</td>
<td>106 (83–135)</td>
<td>86 (46–120)</td>
</tr>
<tr>
<td>Normal serum&lt;sup&gt;a&lt;/sup&gt;</td>
<td>55 (47–65)</td>
<td>65 (62–70)</td>
<td>57 (51–59)</td>
<td>106 (83–135)</td>
<td>86 (46–120)</td>
</tr>
<tr>
<td>RP-HPLC of serum&lt;sup&gt;b&lt;/sup&gt;</td>
<td>50 (45–54)</td>
<td>25 (23–29)</td>
<td>32 (20–43)</td>
<td>96 (70–136)&lt;sup&gt;c&lt;/sup&gt;</td>
<td>61 (34–96)&lt;sup&gt;c&lt;/sup&gt;</td>
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<tr>
<td>GF-HPLC of serum&lt;sup&gt;b&lt;/sup&gt;</td>
<td>37 (24–60)</td>
<td>28 (21–30)</td>
<td>27 (16–39)</td>
<td>35 (21–53)</td>
<td>30 (20–43)</td>
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</tbody>
</table>

<sup>a</sup> Median and 25th and 75th percentile values with the concentrations above the functional sensitivity of the assays included in the comparisons.

<sup>b</sup> Median, minimum, and maximum values of the area under the curve ratios (n = 3–4).

<sup>c</sup> The lowest values correspond to the detection limits of the assays.

NT-proANP<sub>46–79</sub> assays showed significant correlation with each other (r² = 0.82; Fig. 6A). The absolute values, however, varied between these two assays, so that the NT-proANP<sub>1–20</sub> assay consistently yielded ~30% lower concentrations (Table 3). The NT-proBNP<sub>5–24</sub>, NT-proBNP<sub>10–29</sub>, and NT-proBNP<sub>57–76</sub> concentrations also correlated significantly with each other (r² = 0.77–0.85; Fig. 6, C–E), but again the absolute concentrations varied greatly between the assays. The same decrease (30–50%) in the NT-proBNP<sub>57–76</sub> values compared with the NT-proBNP<sub>10–29</sub> and the NT-proBNP<sub>5–24</sub> values was detected in the raw plasma samples and in the HPLC data fractions (Table 3). NT-proBNP (NT-proBNP<sub>5–24</sub>, NT-proBNP<sub>10–29</sub>, NT-proBNP<sub>57–76</sub> and BNP (proBNP<sub>77–108</sub>) values in AMI patients correlated reasonably well (r² = 0.69–0.75; Fig. 6F; data not shown), revealing the same origin of these analytes. NT-proANP and NT-proBNP concentrations of patients with AMI did not, however, show as good a correlation, particularly on admission (r² = 0.26–0.38; Fig. 6B).

**Discussion**

The circulating concentrations of natriuretic peptides and their profragments are now recognized as important indicators of cardiac function. Measurement of these hormones can be useful for distinguishing healthy individuals from patients with different stages of heart failure, for prognosis of long-term survival after heart failure or AMI, and for assessing the treatment of heart failure (20, 32, 33). The specificity and reliability of these assays have, however, been the subjects of debate (24). There is an obvious need for assays that are properly standardized and measure actual endogenous forms of the cardiac hormones (11).

We prepared recombinant plasmids that produce GST fusion proteins of NT-proANP and NT-proBNP to develop homologous assays for the circulating forms of the N-terminal propeptides of ANP and BNP. We also prepared, with the same methods, recombinant proBNP<sub>1–108</sub> and Tyr<sub>0</sub> proBNP<sub>77–108</sub> to aid assay development. GST
Fusion proteins can be produced in high amounts, and they are easy to isolate from *E. coli* lysates by single-step affinity purification (30). The yields were relatively high, although part of the products formed insoluble inclusion bodies. The fusion partner could be easily cleaved from the recombinant target protein by thrombin digestion (31). The pGEX-4T-1 vector contains a cleavage site in the BamHI linker region; therefore, only two additional amino acids are left in the amino terminus of the recombinant protein. We further purified recombinant NT-proANP, NT-proBNP, proBNP1-108, and Tyr-0-proBNP77-108 by RP-HPLC to obtain highly pure peptides for standardization of the RIAs, for tracer preparation, and for calibration of the HPLC analyses. The N-terminal sequencing and mass analyses confirmed that the identity of recombinant NT-proANP corresponded with the full-length GS-NT-proANP1-98. The mass spectrum of recombinant NT-proBNP, however, revealed two species probably corresponding to GS-NT-proBNP1-76 and GS-NT-proBNP1-74. This slight C-terminal truncation has no effect on our quantitative analyses or on the calibration of our assays because our antisera do not bind to this region of the peptide. On the other hand, the presence of the N-terminal Gly-Ser dipeptide so minimally changes the size and hydrophobicity of recombinant proteins that it cannot be detected by the GF-HPLC and RP-HPLC methods used.

We developed two homologous RIAs for NT-proANP and four for NT-proBNP, using high-titer antisera. Each assay uses the appropriate recombinant full-length calibrator but has a different epitope specificity. With these assays the NT-proANP concentration and most of the NT-proBNP concentration can be measured directly in a small amount of plasma or serum without preassay extraction. The analytical ranges of the assays are fairly broad. To our knowledge, this is the first report to describe the production and the use of recombinant NT-proBNP.

Our HPLC data appeared to be consistent with previous finding (25) indicating that NT-proANP circulates in human blood as a single peptide, NT-proANP1-98. On the other hand, our results were clearly at odds with the previous detection of multiple defined fragments of NT-proANP in human circulation (26). We did, however, find some heterogeneity at the NH2 and COOH termini of NT-proANP, which was evidenced by markedly lower concentrations of immunoreactive NT-proANP in the NT-proANP1-20 and NT-proANP79-98 assays compared with the NT-proANP46-79 assay. The difference was particularly clear with serum samples. The terminal fragmentation evidently destroys part of the 1-20 and the 79-98 epitopes. Nevertheless, according to our present results the major part of NT-proANP in human plasma (60-70%) is likely intact NT-proANP1-98.

Previous chromatographic studies have indicated the presence of a high-molecular-mass proBNP peptide (15 kDa) as well as a shorter NT-proBNP peptide (9 kDa) in human plasma (27). It was reported that the 15-kDa...
material could be detected with antisera specific to BNP and NT-proBNP<sub>1-13</sub> but not with antisem specific to NT-proBNP<sub>62-76</sub> (28). The 9-kDa immunoreactive material was recognized by NT-proBNP<sub>1-13</sub> antiserum and less strongly with NT-proBNP<sub>62-76</sub> antiserum. In the present study we used several antisera with epitopes at the N-terminal, middle, and C-terminal regions of NT-proBNP. We had the advantage of being able to use the same recombinant calibrator and tracer in all of the assays. Our results show that the main form of circulating immunoreactive NT-proBNP can be detected by antisera reactive to either of the termini or to the more central region. It is less hydrophobic and has a smaller molecular mass than NT-proBNP; thus, it probably represents NT-proBNP<sub>1-76</sub>-derived peptide(s) truncated at both termini. Our findings that the NT-proBNP<sub>1-22</sub> and NT-proBNP<sub>67-76</sub> assays detect 20–40% less immunoreactivity in this peak compared with the NT-proBNP<sub>10-29</sub> assay support this conclusion. The NT-proBNP immunoreactivity with a smaller molecular mass observed in RP-HPLC analysis of serum samples was recognized only by antisera directed at NT-proBNP<sub>10-29</sub>. Altogether these findings suggest that circulating NT-proANP and especially NT-proBNP are heterogeneous. NT-proBNP fragments into smaller components in the blood, and very little, if any, of the immunoreactive material is intact NT-proBNP<sub>1-76</sub>. The fragmentation is more pronounced in serum than in plasma. In addition, the fragmentation rate detected in this study could be an underestimation because the epitopes of our “terminal” antisera do not reside at the extreme termini of NT-proBNP. We were not able to raise antisera against peptide sequences from the central part of NT-proBNP, which would also bind full-length NT-proBNP; thus, NT-proBNP<sub>10-29</sub> represents the most central epitope tested in the present study. Although NT-proBNP<sub>10-29</sub> was the best epitope tested, we cannot rule out the possibility of slight fragmentation even in this portion of NT-proBNP.

With the NT-proANP<sub>46-79</sub> assay, the range of serum concentrations in healthy individuals was 81–571 pmol/L without any significant difference between the sexes (data not shown). The mean (SD) was 255 (95) pmol/L, which is in accordance with previous reports (12–14, 34). With the NT-proANP<sub>1-20</sub> assay, the mean value was 45% lower, 141 (54) pmol/L. The difference was also seen in the samples from patients with AMI as well as in the HPLC analyses.

The NT-proBNP concentrations in the serum of healthy individuals, as measured with our different assays, ranged from undetectable to 250 pmol/L. We did not detect the previously reported gender divergence (35), although a tendency toward higher values in women could be seen (data not shown). The mean (SD) with the NT-proBNP<sub>10-29</sub> assay was 86 (32) pmol/L. The NT-proBNP<sub>6-24</sub> assay gave almost identical results [86 (41) pmol/L], but with the NT-proBNP<sub>50-79</sub> assay, concentrations were considerably lower [59 (37) pmol/L]. Previously reported plasma concentrations in healthy individuals varied greatly, ranging between 1.5 and 159 pmol/L (16–23). The lowest values were measured with antisera with extreme N-terminal epitopes, and most of the assays required preassay extraction. For example, a fully automated NT-proBNP assay (Roche Diagnostics) uses a pair of antisera raised against NT-proBNP<sub>1-20</sub> and NT-proBNP<sub>39-50</sub> (19). In the light of our data, attention must be paid to fragmentation of proBNP and to the choice of the epitope to be measured. Because truncation of NT-proANP and NT-proBNP mostly takes place in the circulation and during sample storage, assays with critical epitope requirements, such as those with terminal epitopes and sandwich assays, require careful control of preanalytical errors.

We used our newly developed immunoassays to assay samples from cardiac patients to confirm that the results obtained with the assays apply in both physiologic and pathophysiologic situations. In the patients with AMI, the N-terminal peptide concentrations were significantly higher than in healthy individuals, and the development of heart failure among these patients further increased the concentrations. All of our assays could thus identify the postinfarction patients who had reduced cardiac function and heart failure. Previously reported concentrations of NT-proANP in AMI patients varied between 380 and 2500 pmol/L depending on the assay, on the cardiac status of the patient, and on the day of measurement (33–34, 36–38). Typically, NT-proANP has been reported as two- to threefold higher in AMI patients than in healthy persons. Considering that most of our patients belonged to New York Heart Association functional classes 1 and 2, the 1.8- to 2.3-fold increase that we observed is in accordance with the earlier studies. The reported NT-proBNP concentrations in AMI patients have ranged from 96 to 750 pmol/L (7, 35, 39–42). In our study, NT-proBNP concentrations were 4.1- to 4.5-fold higher in AMI patients and 6.6- to 7.8-fold higher in AMI patients with heart failure than in healthy persons. Despite the different absolute concentrations measured by different assays, a threefold difference in NT-proBNP between healthy individuals and AMI patients has been typical in previous studies (24). However, the absolute concentrations were always higher with assays that included a central antisera. According to our results with AMI samples, the extreme N- and C-terminal antisera of NT-proANP and NT-proBNP give ~30–40% lower values than the antisera with more central epitopes. On the basis of these results, we conclude again that the assays with stricter epitope requirements are more prone to preanalytical errors caused, e.g., by differences in sampling and storage. Most, if not all, circulating NT-proANP and NT-proBNP is of cardiac origin (2), regardless of the degree of fragmentation. Therefore, an assay capable of picking the largest proportion of the fragments is likely to best reflect the functional status of the heart.

The quantitative results of our assays for N-terminal ANP and BNP can be compared because the assays use
equivalent native calibrators. In our study, the mean NT-proANP_{10–29} concentration was approximately three-fold higher than the mean NT-proBNP_{10–29} concentration. This resembles the ratios of biologically active ANP and BNP in circulation (1). N-Terminal counterparts of these peptides have longer half-lives (2, 3) and thus higher concentrations in the blood. However, NT-proBNP concentrations in patients with cardiovascular diseases are almost as high as NT-proANP concentrations (2, 24). Because of the low basal concentration of NT-proBNP, the fold change between the samples from healthy individuals and patients is more pronounced with NT-proBNP than with NT-proANP. Accordingly, patients with AMI had 4.5-fold higher concentrations of NT-proBNP_{10–29} than did healthy individuals, whereas the increase in NT-proANP_{46–79} was not more than 1.8-fold. The relatively poor correlation between NT-proANP and NT-proBNP in this pathologic state provides further evidence for the divergent regulation of the two cardiac peptide systems.

In conclusion, we present several immunoassays for NT-proANP and NT-proBNP. All are suitable for direct measurement of the peptides in small volumes of plasma or serum. However, concentrations detected by different assays with various epitope requirements vary because of the heterogeneity of the circulating forms of both NT-proANP and NT-proBNP. Circulating NT-proBNP, in particular, consists of several components of smaller size than intact NT-proBNP. Thus, assays that use antibodies or antisera that are directed at the midregions of NT-proANP or NT-proBNP are the most robust and reliable.

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