The Brain Natriuretic Peptide (BNP) Precursor Is the Major Immunoreactive Form of BNP in Patients with Heart Failure

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Background: Peptides derived from brain natriuretic peptide (BNP) precursor (proBNP), BNP, and the N-terminal fragment of proBNP (NT-proBNP) are used as biomarkers of heart failure. It remains unclear which forms of these peptides circulate in blood and which forms are measured by assays for these natriuretic peptides.

Methods: To design assays for immunodetection of proBNP, NT-proBNP, and BNP, we used a panel of BNP- and NT-proBNP–specific monoclonal antibodies (MAbs). All MAbs were tested in 2-site combinations in time-resolved fluoroimmunoassays with recombinant or synthetic antigens and plasma from heart failure (HF) patients. ProBNP and related molecules were assayed in HF plasma samples and plasma extracts by means of gel filtration fast protein liquid chromatography (FPLC) before and after protein fractionation on Sep-Pak C18 cartridges.

Results: The limits of detection for BNP, proBNP, and NT-proBNP assays were 0.4, 3, and 10 ng/L, respectively. Gel filtration-FPLC studies revealed 1 peak of NT-proBNP (~25 kDa), 1 peak of proBNP (~37 kDa), and 2 peaks of BNP immunoreactivity, a major peak (~37 kDa) for proBNP, and a minor peak (~4 kDa) for BNP. In patient plasma, the molar concentration of NT-proBNP was almost 10 times that of proBNP. The mean proBNP:BNP ratio in patient plasma was 6.3, ranging from 1.8 to 10.8.

Conclusions: ProBNP is the major BNP-immunoreactive form in human blood. The proBNP:BNP ratio in plasma samples is dependent on the methods used for sample handling and for the measurement of the peptides.

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Although both brain natriuretic peptide (BNP)7 and the N-terminal fragment of BNP precursor (NT-proBNP) have been used as markers of heart failure (HF) for more than 10 years, the biochemical peculiarities of these circulating peptides are still poorly understood. Studies based on gel filtration (GF) chromatography in nondenaturing conditions demonstrated that NT-proBNP immunoreactivity is represented by a high–molecular-mass fraction (~25–35 kDa) (1). Similar studies have detected BNP immunoreactivity in 2 fractions, 1 with a molecular mass characteristic of BNP-32 peptide (~3 kDa) and the other with high-molecular-mass, most likely the BNP precursor (proBNP) molecule (~30–40 kDa) (2, 3) with posttranslational modifications (glycosylation) (4). No conclusive evidence exists, however, to elucidate the ratios of the immunoreactive forms of BNP and proBNP in the blood. We sought to better define the molecular forms of proBNP and its derivatives in blood samples of HF patients by use

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7 Nonstandard abbreviations: BNP, brain natriuretic peptide; proBNP, BNP precursor; NT-proBNP, N-terminal fragment of the BNP precursor; HF, heart failure; GF, gel filtration; FPLC, fast protein liquid chromatography; MAb, monoclonal antibody; IFA, immunofluorescent assay.
of a GF-fast protein liquid chromatography (FPLC) separation technique.

Materials and Methods

Materials

Synthetic human BNP with 97% purity according to HPLC studies was obtained from Bachem AG. Human recombinant NT-proBNP and proBNP (expressed in Escherichia coli) with >98% purity according to electrophoresis were from HyTest. Synthetic human atrial natriuretic peptide was from PolyPeptide Laboratories. Synthetic peptides corresponding to sequences 1–10, 11–22, 17–23, and 26–32 of BNP and to 1–12, 5–20, 1–24, 13–27, 28–45, 31–39, 34–42, 37–45, 48–56, 50–58, 52–60, 46–60, 63–71, 65–73, 67–76, and 61–76 of NT-proBNP were also from HyTest. All chemicals were from Sigma.

Preparation of Monoclonal Antibodies

The hybridoma cell lines producing monoclonal antibodies (MAbs) were obtained by use of a standard procedure (5). To obtain NT-proBNP-specific antibodies, mice were immunized with synthetic peptides corresponding to sequences 1–24, 13–27, 28–45, 46–60, and 61–76 of NT-proBNP molecules conjugated to the carrier protein. For BNP MAb production, both synthetic peptides corresponding to sequences 1–10, 11–22, 17–23, and 26–32 of BNP and the whole BNP molecule conjugated to the carrier protein were used as immunogens. Humane treatment of mice was in accordance with the Committee on Care and Use of Laboratory Animals.

Sandwich Immunofluorescent Assay

To perform the sandwich immunofluorescent assay (IFA), we used biotinylated MAbs as capture antibodies and MAbs labeled with stable europium (III) chelate of 

\[
\text{Eu}^{3+}\text{-fplc}
\]

with biotin-isothiocyanate and europium chelate have been described by Katrukha et al. (6). Mixtures of equal quantities (200 ng per well) of biotinylated and Eu\(^{3+}\)-conjugated antibodies in 50 μL of buffer A (0.05 mol/L Tris-HCl, pH 7.7, 9 g/L NaCl, 0.1 mL/L Tween 40, 5 g/L bovine serum albumin, and 0.5 g/L NaN\(_3\)) were incubated in streptavidin-coated plates (Wallac-Perkin-Elmer) with 50 μL of tested sample or the calibrator for 30 min at room temperature with gentle shaking. After washing with buffer B (0.01 mol/L Tris-HCl, pH 7.8, 0.15 mol/L NaCl, 0.25 mL/L Tween 20, and 0.5 g/L NaN\(_3\)) we added 200 μL per well of enhancement solution (1.75 mol/L NaSCN, 1 mol/L NaCl, 50 mol/L glycerol, 200 mL/L 1-propanol, 0.005 mol/L Na\(_2\)CO\(_3\), 0.05 mol/L glycine-NaOH, pH 10.0). The plate was incubated at room temperature for 3 min with gentle shaking, and fluorescence was measured on a Victor 1420 multilabel counter (Wallac-Perkin-Elmer). Total time of the assay was 40 min.

Beckman Access BNP Assay

The Access BNP assay uses a capture MAb specific to the ring structure, with part of the C-terminal fragment of BNP and detection MAb specific to the N-terminal part of the BNP molecule, and enables the detection of both BNP and proBNP (7).

Patients and Blood Samples

Diagnosis of HF was based on patient symptoms of dyspnea, orthopnea, lung rales, or leg edema and confirmed by echocardiography studies (GE Vivid 7 ultrasound imaging system) and x-ray examination. The preliminary diagnosis was made by a cardiologist and further confirmed by an HF expert. Blood samples were collected from patients with left ventricular ejection fractions (measured by modified Simpson’s method) <30% [mean (SD) 23.4%, (5.2%)]) and left-ventricular end-systolic volume >90 mL. Venous blood was collected into EDTA-containing Vacuette tubes (Greiner Bio-One) and centrifuged at 3000g (15 min, +4 °C). Serum samples were obtained from blood collected in plastic tubes, incubated for 30 min at room temperature, and centrifuged at 5000g (30 min, +20 °C). Plasma and serum samples were stored at −70 °C before use. For MAb testing, pooled serum (39 patients with severe HF) or pooled plasma (10 HF patients) was used as a source of endogenous antigens. As a negative (non-HF) control we used pooled serum or plasma from 10 healthy donors. Pooled plasma of healthy donors was also used for dilution of recombinant NT-proBNP, proBNP, and synthetic BNP before applying on GF column and for assay validation. All studies using human blood samples were in accordance with the current revision of the Helsinki Declaration.

Protein Extraction from Plasma Samples

BNP and proBNP were extracted from plasma samples of HF patients by use of Sep-Pak C18 cartridges (Millipore-Waters) according to the method described by Shimizu et al. (2). The eluates were dried for 2–3 h with a reduced pressure evaporator Mini DNA (Heto Lab Equipment).

GF-FPLC

Individual patient plasma samples or Sep-Pak C18 extracts, reconstituted in 0.1 mol/L sodium phosphate buffer, pH 7.4, containing 0.3 mol/L NaCl and 0.005 mol/L EDTA, were centrifuged at 10 000g for 10 min, and supernatants (150 μL) were applied onto Superdex 75 10/300 GL GF column (GE Healthcare) equilibrated with the same buffer. Proteins were eluted at a flow rate of 0.8 mL/min. The column was calibrated with a set of standard proteins (GE Healthcare): albumin (67 kDa), ovalbumin (43 kDa), chymotrypsinogen (25 kDa), ribonuclease A (13.7 kDa), and aprotinin (6.52 kDa). Recombinant NT-proBNP, proBNP, and synthetic BNP were reconstituted in pooled plasma from healthy donors before being loaded onto the Superdex 75 column.
Results

DEVELOPMENT OF AN IMMUNOASSAY FOR QUANTIFICATION OF BNP IN HUMAN BLOOD

Human BNP and BNP fragments conjugated to the carrier protein were used for microparticle immunization. Precise epitope specificity of 65 newly raised MAbs obtained after immunization of mice with the whole BNP molecule was established with the help of the synthetic peptides corresponding to different regions of BNP. All MAbs were able to recognize both synthetic BNP and recombinant proBNP in a direct ELISA. Three of 65 MAbs interacted specifically with peptide 1–10 (amino acid sequence 1–10 of BNP), 17 MAbs interacted with peptide 11–22, 1 MAb interacted with peptide 17–23, and 31 MAbs interacted with peptide 26–32. The epitope specificities of 13 MAbs failed to be established. All BNP-specific MAbs were tested in pairs in sandwich-IFA. Either synthetic BNP or pooled serum (plasma) from HF patients (as a source of circulating native peptide) was used as antigens in this study. MAbs specific to the remote epitopes 11–22 and 26–32 were found to be an efficient 2-site combination for immunodetection of endogenous antigen from patient blood and for the detection of the synthetic BNP.

The antibody combination 50E150–26–32–24C526–32 manifested the highest detection limit in 1-step sandwich-IFA with both synthetic and endogenous antigens and was used to study molecular forms of proBNP and its derivatives in plasma samples of HF patients.

DEVELOPMENT OF AN IMMUNOASSAY FOR QUANTIFICATION OF NT-proBNP IN HUMAN BLOOD

We selected 84 MAbs for their ability to recognize recombinant NT-proBNP in ELISA. Among those, 14 MAbs were specific to region 1–24, 24 MAbs to region 13–27, 19 MAbs to region 28–45, 13 MAbs to region 46–60, and 15 MAbs to region 61–76. Precise epitope mapping of newly generated antibodies was performed by use of a library of synthetic peptides containing overlapping sequences. All MAbs were tested in pairs (capture and detection) in sandwich-IFA with recombinant NT-proBNP or proBNP and with pooled serum or plasma from HF patients as a source of endogenous antigens. All MAbs with remote epitopes being used in 2-site combinations were able to recognize recombinant NT-proBNP and proBNP with a low detection limit (10–100 ng/L). Only a few MAb combinations reacted with the antigen from serum and plasma, however. Thus, none of the 13 MAbs that were specific to region 46–60 and recognized recombinant NT-proBNP were able to recognize endogenous antigen when used in 2-site combinations with any other antibody.

MAbs pairs with 1 antibody specific to region 13–27 and 1 antibody specific to region 61–76 recognized endogenous antigen with high sensitivity. When MAbs specific to the N-terminal region or to region 28–45 were tested in pairs with antibodies recognizing sequence 61–76, the ratio of signals from the endogenous to recombinant peptide was significantly lower than in the assays using MAbs specific to epitopes 13–27. Only MAb 29D12, which is specific to peptide 5–12, elicited high signals upon interaction with either serum or plasma samples being tested in pairs with MAbs specific to peptide 67–76. The assay 15C453–71–13G1213–20 was selected and used in further studies because it exhibited the highest sensitivities to both recombinant and native antigens.

DEVELOPMENT OF AN IMMUNOASSAY FOR QUANTIFICATION OF PROBNP IN HUMAN BLOOD

To develop an assay specific for proBNP, we tested all NT-proBNP- and BNP-specific MAbs in a sandwich-IFA with recombinant proBNP and pooled serum or plasma from HF patients used as antigens. Similarly to their reaction to NT-proBNP in described above studies, MAbs reacted differently with recombinant proBNP and the antigen found in serum or plasma from HF patients. The majority of tested MAb combinations interacted effectively with recombinant proBNP, but far fewer pairs recognized endogenous antigen. Similarly to the previous experiments with NT-proBNP, MAbs against region 28–60 of proBNP (able to recognize recombinant proBNP) interacted only weakly with the antigen in patient serum or plasma being tested in all possible 2-site combinations with other antibodies. Antibody pairs including MAbs specific to region 13–27 of NT-proBNP and MAbs specific to regions 11–22 and 26–32 of BNP elicited the highest signals with endogenous antigen. Among antibodies specific to the N-terminal fragment of NT-proBNP, only 29D12 (peptide 5–12) elicited a relatively high signal when tested in 2-site combinations with endogenous proBNP. In contrast to the NT-proBNP assay, antibodies specific to region 61–76 of NT-proBNP in combination with antibodies against region 11–22 or 26–32 of BNP manifested weak interactions or no interaction with the endogenous antigen but were able to interact with recombinant proBNP.

Among all the antibody combinations tested, the antibody pair 50E1–16F3 manifested the highest sensitivity to recombinant and endogenous proBNP. The 50E1 antibody is directed against site 26–32 of BNP, and MAb 16F3 is specific to peptide 13–20 of NT-proBNP.

IFA VALIDATION

Typical calibration curves for determination of BNP, proBNP, and NTproBNP and serial dilutions of human plasma samples are shown in Fig. 1, A, B, and C, respectively. The detection limits of the assays were 0.4 ng/L for BNP, 10 ng/L for NT-proBNP, and 3 ng/L for proBNP. The detection limit was defined as a concentration, (measured 20 times in a single run) producing a signal 2 SD above the mean for a calibrator that is free of analyte. The BNP assay was linear in the range of 1–200 000 ng/L ($r^2 = 0.9985$). The NT-proBNP assay was linear in the range of 15–100 000 ng/L ($r^2 = 0.9972$), and the proBNP assay was linear in the range of 10–180 000 ng/L ($r^2 = 0.9976$).
Within-assay imprecision (CV) was assessed by measuring 20 replicates of 3 different concentrations of calibrator in pooled normal human plasma and was always <6% (Table 1). Recoveries from pooled patient or individual patient plasma samples, which were enriched with 3 concentrations of analyte, are also shown in Table 1. The

Table 1. IFA validation.

<table>
<thead>
<tr>
<th></th>
<th>BNP 50E1–24C5</th>
<th>NT-proBNP 15C4–13G12</th>
<th>proBNP 50E1–16F3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Detection limit</td>
<td>0.4 ng/L</td>
<td>10 ng/L</td>
<td>3 ng/L</td>
</tr>
<tr>
<td>Linearity range</td>
<td>1–200000 ng/L ($r^2 = 0.9985$)</td>
<td>15–100000 ng/L ($r^2 = 0.9972$)</td>
<td>10–180000 ng/L ($r^2 = 0.9976$)</td>
</tr>
<tr>
<td>Within-assay imprecision (CV, %)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low concentration</td>
<td>4.86 (10 ng/L)</td>
<td>5.42 (100 ng/L)</td>
<td>5.21 (20 ng/L)</td>
</tr>
<tr>
<td>Medium concentration</td>
<td>4.33 (100 ng/L)</td>
<td>5.40 (1000 ng/L)</td>
<td>4.70 (200 ng/L)</td>
</tr>
<tr>
<td>High concentration</td>
<td>4.98 (1000 ng/L)</td>
<td>2.82 (10000 ng/L)</td>
<td>2.67 (2000 ng/L)</td>
</tr>
<tr>
<td>Recovery, %</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low concentration</td>
<td>108.4$^a$; 110.3$^a$; (100 ng/L)</td>
<td>118$^a$; 80.4$^b$ (200 ng/L)</td>
<td>95.5$^a$; 91.5$^b$ (100 ng/L)</td>
</tr>
<tr>
<td>Medium concentration</td>
<td>85.9$^a$; 95.3$^b$ (500 ng/L)</td>
<td>96.3$^a$; 117$^b$ (1000 ng/L)</td>
<td>77.3$^a$; 93.6$^b$ (500 ng/L)</td>
</tr>
<tr>
<td>High concentration</td>
<td>111$^a$; 124$^b$ (5000 ng/L)</td>
<td>109$^a$; 104$^b$ (10000 ng/L)</td>
<td>88.2$^a$; 89.7$^b$ (5000 ng/L)</td>
</tr>
</tbody>
</table>

$^a$ Pooled HF plasma sample.
$^b$ Individual HF plasma sample.
specificity of antibodies used in the assays was confirmed by Western blotting analysis (data not shown). All antibodies detected recombinant proBNP. MAbs 50E1 and 24C5 also detected synthetic BNP, and 15C4, 16F3, and 13G12 also detected recombinant NT-proBNP. Cross-reactivity of atrial natriuretic peptide in the BNP assay was <0.5%.

**GF-FPLC study of plasma samples from HF patients**

Patient diagnoses and peptide concentrations in plasma samples are presented in Table 2. Plasma samples from 7 HF patients were subjected to GF to characterize the molecular forms of proBNP-derived products and to establish their ratios. BNP, proBNP, and NT-proBNP were quantified in fractions with the help of immunoassays 50E1–24C5, 50E1–16F3, and 15C4–13G12, respectively (described above). No preconcentration of specimens was necessary. Mean recoveries in GF studies determined by sandwich-IFA were 68%, 44%, and 59% for BNP, proBNP, and NT-proBNP, respectively.

Fractions analysis by the NT-proBNP assay revealed 1 peak of immunoreactivity with a maximum in fraction 13 (~25 kDa; Fig. 2A). The position of the NT-proBNP activity maximum did not coincide with those established for recombinant NT-proBNP (fraction 15; ~15.3 kDa) and recombinant proBNP (fraction 14; ~20.6 kDa). ProBNP assay measurements of immunological activities in plasma fractions revealed a maximal concentration in fraction 12 (~37 kDa; Fig. 2B). A broad concentration peak with maxima in 12–13 fractions (~25–37 kDa) was observed in the plasma fractions of patient 1.

The analysis of the fractions by the BNP assay revealed the presence of 1 major and 1 smaller peak (Fig. 2C). A minor peak was detected at ~4–6 kDa (fractions 18 and 19; almost coincident with BNP standard) and 1 major peak was detected at ~25–37 kDa [fraction 12 (6 patients); fractions 12 and 13 (1 patient)]. In all plasma samples, the position of the major BNP peak coincided completely with the position of the immunoreactivity peak measured by proBNP assay (Fig. 2B).

**GF-FPLC study of plasma extracts from Sep-Pak C18 column**

To establish the effects of preliminary extraction on the proBNP:BNP ratio, plasma samples from 7 patients described in the aforementioned experiments were applied onto Sep-Pak C18 cartridges. The mean recovery, determined by sandwich-IFA, for this procedure was 30% for BNP and ~7.5% for proBNP. The eluates were dried in evaporator, dissolved in a phosphate-salt buffer, and loaded onto a Superdex 75 column. Fractions were analyzed with our experimental BNP and proBNP assays. BNP immunodetection revealed the presence of 2 peaks of immunological activity (Fig. 2D) in the same fractions as in the experiments with the untreated samples. However, the ratios of the peaks of immunological activities of proBNP and BNP were significantly different from those of nonextracted samples. The values of proBNP:BNP ratio

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**Table 2. Patient diagnoses and peptide concentrations in plasma samples measured in three following assays:**

**15C4–13G12 (NT-proBNP), 50E1–16F3 (proBNP), 50E1–24C5 (BNP) using proBNP as calibrator.**

<table>
<thead>
<tr>
<th>Patient</th>
<th>Diagnosis</th>
<th>NT-proBNP, pmol/L</th>
<th>proBNP, pmol/L</th>
<th>BNP, pmol/L</th>
<th>NT-proBNP/proBNP ratio</th>
<th>BNP/proBNP ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Coronary artery disease: acute anterior ST-elevation myocardial infarction, acute left ventricular dysfunction (pulmonary edema)</td>
<td>6640</td>
<td>705</td>
<td>831</td>
<td>9.4</td>
<td>1.2</td>
</tr>
<tr>
<td>2</td>
<td>Coronary artery disease: ischemic cardiomyopathy, chronic atrial fibrillation, heart failure (NYHA* class IV), cardiac asthma</td>
<td>3392</td>
<td>270</td>
<td>419</td>
<td>12.5</td>
<td>1.5</td>
</tr>
<tr>
<td>3</td>
<td>Coronary artery disease: acute anterior ST-elevation myocardial infarction, postinfarction cardiosclerosis, acute left ventricular dysfunction (pulmonary edema)</td>
<td>4400</td>
<td>440</td>
<td>592</td>
<td>10</td>
<td>1.34</td>
</tr>
<tr>
<td>4</td>
<td>Coronary artery disease: acute inferior ST-elevation myocardial infarction, acute left ventricular dysfunction (pulmonary edema)</td>
<td>3984</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>Coronary artery disease: non-ST-elevation myocardial infarction, ischemic cardiomyopathy, heart failure (NYHA class IV)</td>
<td>397</td>
<td>160</td>
<td>167</td>
<td>2.48</td>
<td>1.04</td>
</tr>
<tr>
<td>6</td>
<td>Coronary artery disease: ischemic cardiomyopathy, essential arterial hypertension, heart failure (NYHA class IV), cardiac asthma</td>
<td>3800</td>
<td>318</td>
<td>355</td>
<td>11.9</td>
<td>1.1</td>
</tr>
<tr>
<td>7</td>
<td>Coronary artery disease: myocardial infarction, heart failure (NYHA class IV), pulmonary edema</td>
<td></td>
<td></td>
<td>560</td>
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</tbody>
</table>

* NYHA, New York Heart Association.
in nonextracted plasma samples and in plasma samples from the same patients measured after extraction on Sep-Pak C18 cartridges are shown in Table 3. The content of proBNP (1st peak) and BNP (2nd peak) were determined and compared. Ratios of immunologically active proBNP and BNP in plasma samples before extraction varied between 1.8 and 10.8 (mean 6.3), whereas in samples subjected to preliminary extraction, ratios were significantly lower, ranging from 0.3 to 3 (mean 1.5).

To demonstrate that the results of the above studies were not dependent on the specificity of our in-house BNP assay, we used a commercial BNP assay (Beckman Access) together with our in-house assay to analyze a representative plasma sample from patient 7 before and after preliminary peptide extraction on Sep-Pak C18 cartridge. The immunological activity profiles of both the commercial and in-house assays (Fig. 3) have identical patterns and differ solely in the absolute values, with higher concentrations measured by the Beckman immunoassay. Similar to the results of fraction testing obtained with our in-house assay, the ratio of proBNP:BNP measured by the Beckman assay was significantly higher in

<table>
<thead>
<tr>
<th>Patient</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patient 1</td>
<td>ProBNP:BNP ratio in plasma samples before extraction</td>
<td>3.9</td>
<td>7.8</td>
<td>1.8</td>
<td>4.2</td>
<td>4.7</td>
<td>10.6</td>
</tr>
<tr>
<td>Patient 2</td>
<td>ProBNP:BNP ratio in plasma samples after extraction on Sep-Pak C18 cartridges</td>
<td>3</td>
<td>0.7</td>
<td>1.3</td>
<td>0.3</td>
<td>2.5</td>
<td>1.7</td>
</tr>
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</table>
plasma before preliminary extraction (Fig. 3A) than in the extracted samples (Fig. 3B).

**Discussion**

In the current study, we used a GF method to separate proBNP and its derivatives circulating in plasma and analyzed them with 3 newly developed, highly sensitive, antigen-specific research IFAs. We show that in HF patient plasma the immunological activity of proBNP is mainly represented by 1 peak corresponding to a peptide with a molecular mass of ~37 kDa. The analysis of the fractions with the BNP-specific assay was able to detect both BNP and proBNP, revealing the presence of 2 peaks. The position of the major peak coincided exactly with the peak of proBNP, whereas the 2nd (minor) peak was localized in the area of 4–6 kDa and was considered to be related to BNP. The ratios of immunoreactive BNP forms (proBNP:BNP) in plasma of HF patients varied from patient to patient, but proBNP content exceeded that of BNP in all cases (by 1.8- to 10.8-fold). On the basis of these findings, we concluded that proBNP was the major antigen contributing to immunological activity measured by both the Beckman commercial assay and our experimental BNP assay in HF patients.

To develop reliable and highly sensitive assays for precise and specific detection of proBNP and proBNP-derived peptides, we generated large panels of MAbs specific to NT-proBNP and BNP, with epitopes covering the whole sequences of both molecules. As a source of endogenous protein for pair testing we used not only synthetic/recombinant antigens but also serum and plasma samples from HF patients. This approach helped us resolve 2 issues. First, we identified the epitopes that are changed or modified in the endogenous antigens compared with synthetic/recombinant molecules. Second, we identified MAb combinations that recognized endogenous molecules with the same sensitivity and efficiency as their “artificial” counterparts. We demonstrated that MAbs specific to the central part (region 28–60) of the NT-proBNP molecule are almost unable to recognize the antigen in human blood, a phenomenon that may be attributable to posttranslational modification(s) of endogenous NT-proBNP. We have since learned that the central portion of the NT-proBNP molecule is glycosylated, and polysaccharide residues prevent antibodies from interaction with the endogenous antigen. Schellenberger et al. (4) reported that in CHO cells proBNP is expressed as a glycoprotein. Several sites of glycosylation located in the central part of the molecule were documented. It was also demonstrated that proBNP in patient blood is glycosylated. Because antibodies specific to the central, glycosylated part of the NT-proBNP molecule are unable to recognize endogenous antigen, MAbs specific to other regions not affected by glycosylation should be used in NT-proBNP and proBNP assays.

For many years chromatography was the prevailing method of analysis of proBNP, NT-proBNP, and BNP circulating in human blood. Because of the relatively low sensitivity of the immunoassays used to quantify antigens in the samples after chromatography, concentration of the sample before applying it onto column was necessary to compensate for the dilution during chromatography (2, 3, 8).

Previous studies revealed that plasma samples from HF patients contain significant amounts of proBNP. In some samples proBNP concentration was even greater than that of BNP, but usually reported proBNP:BNP ratios were not >2 (2, 3). We observed significantly higher differences between proBNP and BNP concentrations than those previously reported. In our studies the proBNP:BNP ratio varied from 1.8 to 10.8, with a mean of 6.3. The major experimental difference between our study and previous studies was that we skipped the step of plasma extraction/concentration before applying the sample onto the GF column. The high sensitivity of our research assays allowed us to quantify analytes even in

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Fig. 3. The immunological activity of the plasma fractions (patient 7) obtained after GF on Superdex 75 as measured by 50E1–24C5 assay (♦) or by the commercial reagent set BNP Beckman Access (■). (A), without preliminary extraction; (B), after preliminary extraction of the plasma sample on a Sep-Pak C18 cartridge. The positions of standard proteins used for the calibration of the GF column are marked by arrows.
the diluted samples after GF-FPLC, leading us to assume that preliminary extraction of the proteins might change proBNP:BNP ratios. To test this hypothesis, we compared the concentrations of both analytes in plasma samples before and after extraction on Sep-Pak C18 cartridges performed according to Shimizu et al. (2). We demonstrated that proBNP:BNP ratios in the samples before extraction and after extraction on Sep-Pak C18 differed significantly. In nonextracted samples, the mean ratio was ~6.3, whereas in the extracted samples, molar concentrations of both peptides were almost equal (mean 1.5). The reason for the apparent increase in the magnitude of the BNP peak (relative to proBNP) should be sought in the incomplete elution of proBNP (a significantly bigger molecule than BNP) from Sep-Pak C18 cartridges. We found that extraction changes the peptide ratio significantly, decreasing apparent proBNP content in the sample. Thus we feel that studies of BNP and proBNP content in patient plasma that are performed without preliminary peptide extraction are more relevant, and the results of our study better reflect the real concentrations and/or ratios of analytes.

To verify that differences in proBNP:BNP ratios were not biased by the specificity of our in-house BNP assay, we analyzed 1 of the plasma samples by both a commercial BNP assay (Beckman Access) and our in-house BNP assay (Fig. 3). For both assays the results of BNP immunoreactivity measurements were very similar. When the sample was not subjected to the preliminary extraction, both assays showed 2 activity peaks: the major peak corresponded to proBNP, and the minor peak almost corresponded to BNP. The proBNP:BNP ratios were 8.6 (Beckman) and 10.8 (in-house). After preliminary extraction, the ratio changed: the peak heights of proBNP and BNP immunoreactivities became practically identical (proBNP:BNP ratio 0.9 Beckman, 1.09 in-house). It should be also noted that our data are in good agreement with recently published results by Hawkridge et al. (9), who observed no detectable free BNP in HF patient plasma. Quantitative differences of our data vs findings of Hawkridge et al. (9) could be explained by methodological differences.

In conclusion, our data suggest that proBNP is the predominant form displaying BNP immunoreactivity in patients with HF. We postulate that direct proBNP measurements by assays, using one antibody specific to the BNP and another to the NT-proBNP part of the molecule, may be of clinical and commercial value. Additional studies of large cohorts of HF patients will be required to obtain experimental evidence for these statements.

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