Novel Immunoassay for Quantification of Brain Natriuretic Peptide and Its Precursor in Human Blood

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BACKGROUND: Brain natriuretic peptide (BNP) is an unstable molecule that can rapidly lose immunologic activity in blood. Conventional sandwich BNP immunoassays use 2 antibodies specific to 2 different epitopes. Larger distances between epitopes are associated with a greater probability of proteolysis sites being located between the antibody-binding sites, and thus such assays have an increased susceptibility to underdetect BNP because of the increased likelihood of proteolytic degradation. The purpose of our study was to develop a sandwich immunoassay for the precise quantification of BNP and BNP precursor (proBNP) in human blood that is not susceptible to proteolysis.

METHODS: Mice were immunized with an immune complex consisting of monoclonal antibody (MAb) 24C5 (specific for BNP peptide 11–22) and the entire BNP molecule. The MAb used in our assay (Ab-BNP2) recognizes the immune complex but neither free BNP nor MAb 24C5.

RESULTS: We used MAbs 24C5 and Ab-BNP2 to develop a new type of sandwich BNP assay (the “single-epitope sandwich assay”), which requires only a short BNP fragment (fragment 11–22) for immunodetection. This assay recognizes both BNP and proBNP with the same efficiency and sensitivity and demonstrates both considerably less susceptibility to antigen degradation and greater stability of the measured antigen than conventional sandwich BNP immunoassays.

CONCLUSIONS: We have developed this sensitive single-epitope sandwich assay for detecting BNP, proBNP, and their fragments in human blood. This assay appears promising for use in clinical studies to assist in triage, management, and outcomes assessment in heart failure patients.

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Several commercial assays have been developed for the quantitative immunodetection of brain natriuretic peptide (BNP)7 and the N-terminal fragment of the BNP precursor (proBNP) in human blood and are widely used in clinical practice. Most BNP assays have been designed as sandwich assays that use 2 monoclonal antibodies (MAbs) specific for different epitopes. At least one of the 2 antibodies is specific for the ring structure of the BNP molecule, whereas the other can be specific for the C terminus of the peptide [Abbott AxSYM and Architect (1), Bayer ADVIA Centaur (2), Shionogi IRMA (3)] or to the N terminus [Biosite Triage (4), Beckman Access (5)]. The purpose of our study was to develop a new type of immunoassay, which we have designated as the “single-epitope sandwich assay,” for quantifying BNP and proBNP in human blood. This assay has the following features: (a) the capture antibody recognizes an antigen, whereas the detection antibody is specific to the immune complex of the capture antibody and the antigen; and (b) the assay does not recognize the individual components of the immune complex.

Materials and Methods

Synthetic BNP (32 amino acid residues) with an HPLC-documented purity of >96% was obtained from Peptide Institute and Bachem. HyTest provided the following: BNP-specific MAbs 24C5 (epitope 11–22 HyTest Ltd., Turku, Finland;2 Department of Biochemistry, Moscow State University, Moscow, Russia; 3 Moscow Research Institute of Medical Ecology, Moscow, Russia; 4 67 City Hospital, Moscow, Russia; 5 Moscow State Medicosomatological University, Moscow, Russia; 6 Hennepin County Medical Center, University of Minnesota School of Medicine, Minneapolis, MN.

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1 Nonstandard abbreviations: BNP, brain natriuretic peptide; proBNP, BNP precursor; MAb, monoclonal antibody; HF, heart failure.
22), 57H3, and 50E1 (epitope 26–32); synthetic peptides corresponding to sequences 11–22 and 26–32 of the BNP molecule; human recombinant proBNP (expressed in Escherichia coli); and human recombinant glycosylated proBNP (expressed in the HEK 293 cell line). Synthetic human atrial natriuretic peptide and C-type natriuretic peptide were obtained from Bachem. Pooled nonpathologic human EDTA plasma was used as the matrix in stability studies of proBNP, or MAb 24C5 as the antigen for the plate coating. BNP (50 ng per 100 µL), BNP conjugated with carrier protein (ovalbumin), proBNP (expressed in E. coli), or MAb 24C5 (100 ng per 100 µL) was added to the wells in PBS (10 mmol/L KH2PO4, 150 mmol/L NaCl, pH 7.4) and incubated for 30 min at room temperature with gentle shaking. MAb Ab-BNP2 conjugated with stable Eu3⁺ chelate (for conjugation method see (7)) in buffer A (0.05 mol/L Tris-HCl, pH 7.7, 9 g/L NaCl, 0.1 mL/L Tween 40, 5 g/L BSA, and 0.5 g/L NaN3) was added to wells that had previously been washed 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 NaN3). After a 30-min incubation and washing with buffer B, we added 200 µL of enhancement solution (1.75 mol/L NaSCN, 1 mol/L NaCl, 50 mL/L glycerol, 200 mL/L 1-propanol, 0.005 mol/L Na2CO3, 0.05 mol/L glycine/NaOH, pH 10.0) to each well, incubated the plate at room temperature for 3 min, and then measured the fluorescence with a Victor 1420 multilabel counter (Wallac/PerkinElmer). Wells coated with MAb 57H3, which is specific to region 26–32, were used to ensure that BNP orientation did not influence antigen recognition by MAb Ab-BNP2 (MAb 57H3, 1 µg per 100 µL; BNP, 5 000 ng/L).

**CONVENTIONAL BNP SANDWICH IMMUNOFLUORESCENCE ASSAY**

The conventional BNP assay used in this study was previously described by Seferian et al. (8). In brief, biotinylated MAb 50E1 (epitope 26–32) was used as the capture antibody, and MAb 24C5 (epitope 11–22) labeled with stable Eu3⁺ chelate was used as the detection antibody. Mixtures of equal quantities of biotinylated and Eu3⁺-labeled antibodies (200 ng per well) in 50 µL of buffer A were incubated in streptavidin-coated plates with either test sample or calibrators for 30 min at room temperature with gentle shaking. After washing, the signal was detected as described above.

**SINGLE-EPITOPE SANDWICH IMMUNOFLUORESCENCE ASSAY**

Biotinylated MAb 24C5 (epitope 11–22) was used as a capture antibody to form a first-order immune complex with an antigen (BNP or proBNP), whereas MAb Ab-BNP2 labeled with stable Eu3⁺ chelate was used as the detection antibody for the first-order immune complex. Mixture of equal quantities of biotinylated and Eu3⁺-conjugated antibodies (200 ng per well) in 50 µL of buffer A were incubated in streptavidin-coated plates with a test sample or a calibrator for 30 min at room temperature with gentle shaking. After washing with buffer B, we added enhancement solution (200 µL per well) and measured the fluorescence. The detection...
limit was defined as the concentration (measured 20 times in a single run) that produces a signal 3 SDs above the mean for a matrix free of the analyte. Within-assay imprecision (CV) was assessed by measuring 20 replicates each of 3 different concentrations of calibrator [BNP: 10, 100, and 1000 ng/L (Peptide Institute); proBNP: 30, 300, and 3000 ng/L] spiked into buffer A. Total imprecision (CV) was determined by measuring 3 replicate samples at each of 3 proBNP concentrations (100, 1000, and 10 000 ng/L) in buffer A daily for 5 days. In recovery studies, 3 concentrations of proBNP (100, 1000, and 10 000 ng/L) were spiked into 2 individual plasma samples from HF patients with medium and high endogenous proBNP concentrations (2498 ng/L and 13 960 ng/L, respectively).

**STABILITY STUDIES**

We spiked synthetic BNP (Bachem) at a concentration of 10 000 ng/L into pooled nonpathologic human EDTA plasma containing 0.1% sodium azide as a preservative to prevent bacterial growth and incubated the samples at 25 °C for different periods up to 92 h. We also incubated 2 individual plasma samples from HF patients (3763 ng/L and 4126 ng/L of endogenous BNP as measured with the 24C5–Ab-BNP2 assay) at 25 °C for different times up to 72 h. At the end of the incubation period, each sample was immediately frozen at −70 °C and stored at this temperature for not more than 2 weeks before use. The apparent stability of the synthetic antigen was measured with 2 in-house assays (50E1–24C5, 24C5–Ab-BNP2) and with the Abbott Architect BNP assay, whereas the apparent stability of the endogenous antigen was measured with the 2 in-house assays only.

**GEL-FILTRATION STUDIES OF PLASMA SAMPLES**

A Superdex Peptide column (GE Healthcare) was washed with 0.1 mol/L potassium phosphate buffer, pH 7.4, containing 0.7 mol/L NaCl and 5 mmol/L EDTA. We loaded 6 individual plasma samples from HF patients (0.5 mL of each sample) onto the column, separated the proteins at a pump speed of 0.8 mL/min,
and collected 0.4-mL fractions (ÄKTApurifier FPLC system, GE Healthcare). The column was calibrated with ribonuclease A (13.7 kDa), aprotinin (6.5 kDa), a synthetic peptide corresponding to amino acid residues 37–60 of interleukin-1 receptor-like 1 (2.9 kDa), and vitamin B12 (1.35 kDa).

Results

DEMONSTRATION OF MAb Ab-BNP2 SPECIFICITY

Fig. 1 summarizes the results of MAb Ab-BNP2 testing in the direct ELISA with BNP (Bachem) (Fig. 1A) or proBNP (Fig. 1B) used as the antigen for plate coating, and with the BNP incubated on a plate precoated with MAb 57H3 (Fig. 1C), which is specific to region 26–32 of the BNP molecule. Control MAb 24C5, which is specific to BNP region 11–22, recognizes proBNP (used for the plate coating) with greater efficiency than BNP. When BNP was used for the plate coating, region 11–22 was not accessible by the antibodies because this region participates in forming contacts with the plate surface. This is the reason for the different ordinate scales in Fig. 1, A and B. MAb Ab-BNP2 did not recognize either free BNP (not bound by MAb 24C5) or free proBNP. MAb Ab-BNP2 does not recognize BNP coupled to the carrier protein (see Fig. 1 in the Data Supplement that accompanies the online version of this article at http://www.clinchem.org/content/vol54/issue9). MAb Ab-BNP2 also did not recognize MAb 24C5, which was used for the plate coating in the direct immunoassay (data not shown). To demonstrate that MAb Ab-BNP2 could recognize the immune complex of 24C5 with BNP (or proBNP or peptide 11–22), we used MAb 24C5 for the plate coating and incubated the plates with BNP (Bachem) or the synthetic peptide corresponding to amino acids 11–22 of the BNP sequence. The single-epitope sandwich immunoassay recognized BNP and peptide 11–22 with comparable efficiency. The control assay with the MAb 57H3 detection antibody (specific to region 26–32 of the BNP molecule) recognized BNP but did not recognize peptide 11–22 (see Fig. 2 in the online Data Supplement).

SINGLE-EPITOPE SANDWICH ASSAY VALIDATION

The single-epitope sandwich assay recognizes 3 BNP immunoreactivity forms [BNP, recombinant proBNP (nonglycosylated, E. coli), and recombinant proBNP (glycosylated, HEK 293 cell line)] with the same efficiency. Typical calibration curves for BNP, proBNPs, and serial dilutions of human plasma samples from HF patients are shown in Fig. 2. Our assay-validation results for detection limit, assay linearity range, within-assay imprecision, total imprecision, recovery studies, and cross-reactivity are summarized in Table 1.

Fig. 2. Recognition of different antigen forms by the single-epitope sandwich assay.

(A), Calibration curves for 3 different antigens (Ags): synthetic BNP (●), glycosylated recombinant proBNP (HEK 293 cell line) (□), and recombinant nonglycosylated proBNP (E. coli) (▲). The linearity range for the 3 Ags was 0.00023–17.6 nmol/L, which corresponds to a range of 0.8–60 000 ng/L for BNP and 2.7–210 000 ng/L for proBNP. (B), Calibration curve with BNP as the calibrator (●) and serial HF plasma dilutions (●, ▲, □).

STABILITY STUDIES

Synthetic BNP demonstrated very low stability in the conventional BNP assays (50E1-24C5 and Abbott Architect). There was virtually complete loss of BNP immunoreactivity in the sample after 8 h of incubation at 25 °C (Fig. 3A). About 80% of the initial BNP immunoreactivity was detected after 72 h of incubation in samples containing endogenous antigen when they were measured with the in-house 50E1-24C5 assay (Fig. 3B). The stability of both forms of the antigen was significantly higher when it was measured with the single-epitope sandwich assay. Approximately 75% and 40% of the initial immunoreactivity of the synthetic antigen were detected by the single-epitope sandwich assay in the sample after incubation at room tempera-
BNP IMMUNOREACTIVITY MEASUREMENTS IN FRACTIONS AFTER GEL-FILTRATION SEPARATION OF PLASMA PROTEINS OF HF PATIENTS

We used 2 assays (50E1-24C5 and 24C5–Ab-BNP2) to measure BNP immunoreactivity in gel-filtration fractions. Both assays detected 2 peaks of BNP immunoreactivity. The high molecular weight peak (fractions 7–14) was identified as proBNP, and the low molecular weight peak (fractions 18–23) was identified as BNP. Fig. 4 shows BNP immunoreactivity measurements for the 2 assays as a percentage of the fraction containing the highest proBNP concentration. The proBNP/BNP ratio varies in each patient sample and depends on the type of immunoassay used. The proBNP/BNP ratio for the conventional assay varied from 10.6 to 32.6 (mean, 23.9), whereas the ratio for the single-epitope sandwich assay varied from 3.9 to 16.3 (mean, 6.5). The total BNP amounts measured in fractions 18–23 with the single-epitope sandwich assay (0.13–0.37 ng; mean, 0.22 ng) were 2- to 5-fold greater than those obtained with the conventional assay (0.03–0.13 ng; mean, 0.06 ng). The quantities of proBNP and BNP and the proBNP/BNP ratios obtained with the 2 types of BNP assays for plasma samples from 6 HF patients are presented in Table 1 in the online Data Supplement.

CORRELATION WITH THE ABBOTT ARCHITECT ASSAY

Sixty samples from HF patients with BNP concentrations of 965–48 600 ng/L (results were obtained with the conventional BNP 50E1-24C5 assay) were analyzed with the single-epitope sandwich assay and the Abbott Architect BNP assay (Fig. 5). The Architect BNP findings were >5-fold lower than those obtained with the single-epitope sandwich assay ($r = 0.95$).

Discussion

BNP is a useful clinical marker for assisting in the triage and management of HF patients. For 2 decades scientists have tried to improve on the accuracy of BNP quantification by developing new generations of immunoassays. Present-day immunoassays are mostly sandwich-type assays that use 2 antibodies specific for 2 different epitopes. Sandwich assays are sensitive, antigen specific, and rapid and have a broad measurement range. Developing sandwich immunoassays for small molecules such as BNP, which has only 32 amino acid residues is challenging, however. Eleven (>30%) of these 32 residues are identical with those of 2 other peptides, atrial natriuretic peptide and C-type natriuretic peptide.

### Table 1. Single-epitope sandwich assay validation.

<table>
<thead>
<tr>
<th>Limit of detection</th>
<th>0.4 ng/L (determined for BNP form)</th>
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<tbody>
<tr>
<td>Linearity range</td>
<td>0.00023–17.6 nmol/L (antigens: BNP, proBNPs; $r^2 = 0.999$)</td>
</tr>
<tr>
<td>Within-assay imprecision (CV), %</td>
<td></td>
</tr>
<tr>
<td>Low concentration</td>
<td>2.3 (proBNP, 30 ng/L), 7.2 (BNP, 10 ng/L)</td>
</tr>
<tr>
<td>Medium concentration</td>
<td>3.7 (proBNP, 300 ng/L), 3.7 (BNP, 100 ng/L)</td>
</tr>
<tr>
<td>High concentration</td>
<td>5.4 (proBNP, 3000 ng/L), 5.0 (BNP, 1000 ng/L)</td>
</tr>
<tr>
<td>Total imprecision (CV), %</td>
<td></td>
</tr>
<tr>
<td>Low concentration</td>
<td>9.2 (proBNP, 100 ng/L)</td>
</tr>
<tr>
<td>Medium concentration</td>
<td>9.0 (proBNP, 1000 ng/L)</td>
</tr>
<tr>
<td>High concentration</td>
<td>9.2 (proBNP, 10 000 ng/L)</td>
</tr>
<tr>
<td>Recovery, %</td>
<td></td>
</tr>
<tr>
<td>Low concentration</td>
<td>101.7, a 98.5b (proBNP, 100 ng/L)</td>
</tr>
<tr>
<td>Medium concentration</td>
<td>99.8, a 100.3b (proBNP, 1000 ng/L)</td>
</tr>
<tr>
<td>High concentration</td>
<td>93.4, a 94.1b (proBNP, 10 000 ng/L)</td>
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<td>Cross-reactivity</td>
<td></td>
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<tr>
<td>ANP (1000 ng/L)</td>
<td>BNP value, 0.13 ng/L</td>
</tr>
<tr>
<td>CNP (1000 ng/L)</td>
<td>BNP value, 0.00 ng/L</td>
</tr>
</tbody>
</table>

* Results for the plasma sample from HF patient 1.

* Results for the plasma sample from HF patient 2.

a ANP, atrial natriuretic peptide; CNP, C-type natriuretic peptide.
uric peptide, that belong to the same hormone group. Only 3 epitopes for MAbs (at the molecule’s N terminus, the middle fragment, and the C terminus) have been described in the literature (5, 9).

Another problem associated with BNP immunodetection is the stability of the endogenous antigen in whole blood or plasma, and the published data are inconsistent in this respect (2, 9–13). Taken together, these data support the fact that exogenous (usually synthetic) antigen spiked into whole blood or plasma is extremely unstable (9, 14).

We have described a new type of BNP assay. It can be regarded as a modification of a sandwich immunoassay but is distinguished from the conventional sandwich assay by requiring only one epitope for antigen immunodetection by 2 different antibodies (hence our appellation for this BNP assay, the single-epitope sandwich assay). One of the 2 antibodies (MAB 24C5) is used as a capture antibody and is specific for the region consisting of BNP amino acid residues 11–22 (\texttt{11FGRKMDRISSSS22}). The second MAB (Ab-BNP2) is used as the detection antibody and recognizes the immune complex of the first antibody with the antigen. The second antibody does not recognize the free antigen. Only the primary immune complex consisting of MAB 24C5 and BNP, which forms the proper epitope de novo, serves as the antigen for Ab-BNP2.

Although only one antibody-binding site is used in the single-epitope sandwich assay for antigen detection, such assays have major advantages over conventional sandwich assays, including high sensitivity, high specificity, an apparently short assay time, and a broad linearity range. The incubation time was 30 min in our experiments, but this time can be reduced with a smaller incubation volume. This possibility makes the single-epitope sandwich assay applicable for point-of-care assay systems. The broad linearity range (0.00023–17.6 nmol/L) enables measurements of antigen concentrations in both healthy and severely ill patients without special sample pretreatment or dilution. Serial dilutions of plasma samples from HF patients (Fig. 2B), along with our demonstration of similar responses to the different forms of the antigen (synthetic, recombinant glycosylated, and recombinant nonglycosylated; Fig. 2A), show that the single-epitope sandwich assay recognizes synthetic antigen with the same efficiency as the endogenous antigen. This evidence supports our suggestion that the single-epitope sandwich assay may...
be an optimal biomarker assay for quantifying all forms of BNP in a patient’s blood, the substantial clinical implications of which have yet to be ascertained.

Stability studies have revealed that the single-epitope sandwich assay has one very important advantage over the conventional assay: an extraordinary nonsusceptibility to proteolytic degradation of the antigen. BNP stability with the synthetic antigen is significantly higher in the single-epitope sandwich assay than in the conventional assay. Approximately 75% of BNP immunoreactivity was retained with our assay after an 8-h incubation at 25 °C, whereas BNP immunoreactivity was virtually absent when the conventional immunoassay was used. With the endogenous BNP antigen, the apparent decrease in BNP immunoreactivity measured with the conventional assay was much less. BNP immunoreactivity in the plasma of HF patients has recently been shown to be mostly represented by the glycosylated proBNP form, with the proBNP/BNP ratio varying from patient to patient (8, 15). According to our previously published data (16), glycosylated proBNP (endogenous and recombinant) is very stable. We suggested that the apparently higher stability of the endogenous antigen compared with the synthetic antigen could be explained (at least partially) by the greater stability of glycosylated proBNP (the major form of BNP immunoreactivity in human blood) compared with synthetic BNP. We postulate that the greater the distance between epitopes and the larger the epitopes of the antibodies used in the conventional assay, the more cleavage sites that can occur between and within epitopes. In the single-epitope sandwich assay, both antibodies use only one epitope to form the immune complex. Accordingly, the apparent stability of BNP was significantly greater with the single-epitope sandwich assay than with 2 conventional assays, BNP 50E1-24C5 and the Abbott Architect BNP assay. The amounts of BNP measured with the single-epitope sandwich assay in fractions obtained after gel-filtration HPLC of plasma proteins from HF patients were 2- to 5-fold higher than those obtained with a conventional assay. Guided by these observations, we have concluded that a substantial portion of the BNP molecule in human blood circulates as protease-degraded fragments. The conventional assay, which uses 2 different distant epitopes, recognizes only a small proportion of these fragments, whereas the single-epitope sandwich assay detects any molecule fragment containing the epitope of the 24C5 antibody. Thus, by minimizing the influence of protease activity, the single-epitope sandwich assay measures the actual content of BNP immunoreactive forms in a patient’s blood more accurately than conventional assays.

The correlation of BNP measurements in plasma samples from 60 HF patients obtained by conventional assays with those obtained with the single-epitope sandwich assay was acceptable but not ideal. The different calibrators used in the Architect and the single-epitope sandwich assays can account for the observed proportional differences; however, we also suggest that the difference in the epitope specificities of the antibodies used in the assays is another possibility. The Architect assay uses 2 MAbs, a capture antibody from Scios (anti-BNP 106.3) that recognizes the ring structure and possibly part of the arm extending to the C terminus and a detection antibody (anti-BNP BC203) from Shionogi that is specific to the C terminus of the molecule (ARCHITECT® BNP Assay Performance Verification, AACC 2006). The epitope specificity of the antibodies used in the Architect assay resembles the epitope specificity of the antibodies used in 50E1-24C5 HyTest in-house BNP assay. We can assume (and stability studies confirm this hypothesis) that the Architect assay is more susceptible to proteolytic degradation of BNP than the single-epitope sandwich assay. The greater susceptibility to proteolysis of the Architect
and 50EI-24C5 HyTest assays produces greater variation in BNP antigen measurements than obtained with our assay.

In summary, we have described a new type of immunosassay, the single-epitope sandwich assay, that is applicable to human blood samples for the precise detection of very small and unstable BNP molecules. We suggest that such an assay could be useful for the immunodetection of larger antigens with a very limited number of unique epitopes that are different from structurally similar molecules.

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