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ProBNP is a Major Form of BNP Immunoreactivity in Blood of Patients with Heart Failure

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INTRODUCTION

Brain natriuretic peptide (BNP) is a peptide hormone possessing natriuretic, vasodilatory and renin inhibitory properties. BNP arises as a result of proteolytic processing of the precursor molecule proBNP (108 amino acid residues (aar)). ProBNP is cleaved by unknown protease forming two peptides – BNP (aar 77-108) and N-terminal part of the proBNP (aar 1-76). Both BNP and NT-proBNP as well as unprocessed proBNP are secreted into the bloodstream and circulate in human blood.
BNP is widely used in clinical practice for the diagnosis of heart failure (HF), risk stratification of patients with various cardiac pathologies and therapy monitoring. However it remains unclear what forms of this peptide could be found in circulation and what exactly BNP assays detect in human blood. The purpose of the current study was to define better the molecular forms of proBNP derivatives and to establish the ratio of BNP and proBNP in HF patients' blood.

HyTest

Materials and Methods

Monoclonal antibodies: MAb 50E1 (specific to epitope 26-32 of human BNP molecule), MAb 24C5 (specific to epitope 11-22 of human BNP molecule) and MAb 16F3 (specific to epitope 13-20 of human NT-proBNP molecule) were from HyTest (Finland).

Antigens: Synthetic human BNP was purchased from Bachem AG. Recombinant human proBNP expressed in *E. coli* was from HyTest.

Plasma samples: Venous blood from HF patients and healthy subjects was collected into K_3EDTA-containing Vacuette tubes (Greiner Bio-One) and centrifuged at 3000g (15 min, +4°C). Plasma samples were stored at -70°C prior to use.

Gel-Filtration FPLC: 7 HF patients' plasma samples (150 µL) were applied onto Superdex 75 10/300 GL gel filtration column (GE Healthcare) equilibrated with 0.1 mol/L sodium phosphate buffer pH 7.4 containing 0.3 mol/L NaCl and 0.005 mol/L EDTA. Proteins were eluted at a flow rate of 0.8 mL/min. The column was calibrated with a set of standard proteins (GE Healthcare). Recombinant proBNP and synthetic BNP were reconstituted in pooled plasma from healthy donors before being loaded onto the Superdex 75 column. BNP and proBNP were quantified in fractions with the help of in-house immunoassays and Beckman Access BNP assay.

BNP and proBNP sandwich immunofluorometric assays: To design BNP immunoassay we used biotin-conjugated MAb 50E1 as capture and MAb 24C5 labeled with stable Eu^{3+} chelate as detection antibody. In proBNP assay biotin-conjugated MAb 50E1 was also used as capture and NT-proBNP-specific MAb 16F3 labeled with stable Eu^{3+} chelate – as detection antibody.

Mixtures of equal quantities (200 ng per well) of biotin- and Eu^{3+} -conjugated antibodies in 50 µL of assay buffer were incubated in streptavidin-coated plates (Wallac-Perkin Elmer) with 50 µL of tested sample or calibrator for 30 min at room temperature with gentle shaking. After plate washing 200 µL of the solution enhancing fluorescent signal was added per well. 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).

In Fig. 1 we present the calibration curves for 50E1-24C5 (BNP) and 50E1-16F3 (proBNP) immunoasays. The detection limits of the assays were 0.4 ng/L for BNP and 3 ng/L for proBNP assay. The BNP assay was linear in the range of 1-200 µg/L and the proBNP assay linear in the range 10-180 µg/L.

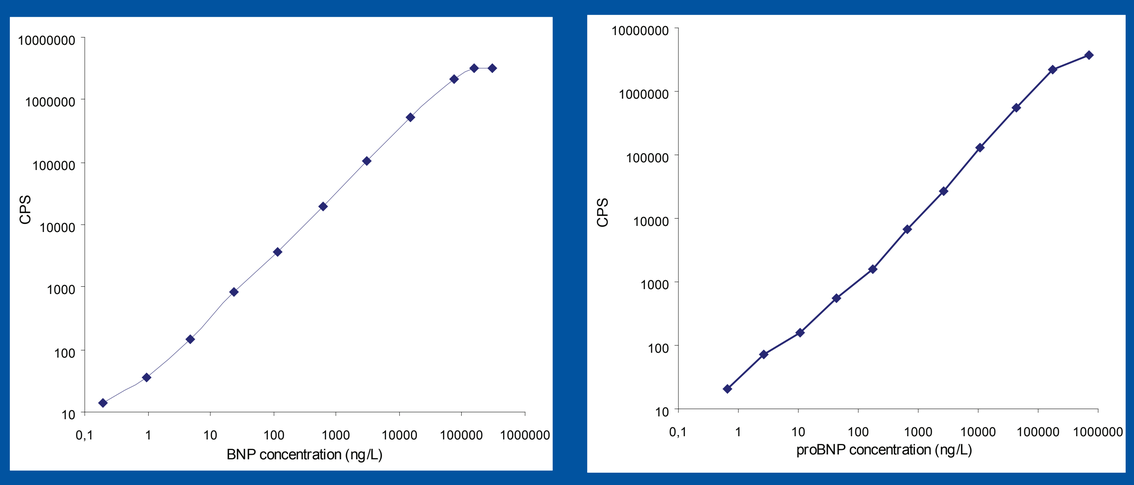

Figure 1: Calibration curves for BNP 50E1 – 24C5 (A) and 50E1-16F3 (B) sandwich fluoroimmunoassays.

Results and Discussion

In the current study we used gel filtration method to separate proBNP and its derivatives circulating in plasma and analyzed them with 2 highly sensitive, antigen-specific immunofluorescent assays. It was show that in HF patients' plasma the immunological activity of proBNP is mainly represented by 1 peak corresponding to a peptide with a molecular mass of ~37 kDa (fraction #12, Fig. 2A). BNP measurements with the BNP-specific immunoassay revealed the presence of 2 peaks. The position of the major peak coincided exactly with the peak of proBNP, whereas the 2nd (minor) peak was in the area of 4-6 kDa (fractions #18 and 19, Fig. 2B) and we considered it to be related with 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 with the mean 6.3).

To verify that differences in proBNP:BNP ratios were not biased by the specificity of our in-house BNP assay, we analyzed fractions after gel filtration (one of the plasma sample) by Beckman Access BNP assay (Fig. 3). Results of BNP immunoreactivity measurements by our in-house and Beckman Access assays were very similar. Beckman BNP assay also showed 2 immunoreactivity peaks with the major peak corresponding to proBNP, and the minor peak almost corresponded to BNP. The proBNP:BNP ratios were 8.6 (Beckman) and 10.8 (in-house).

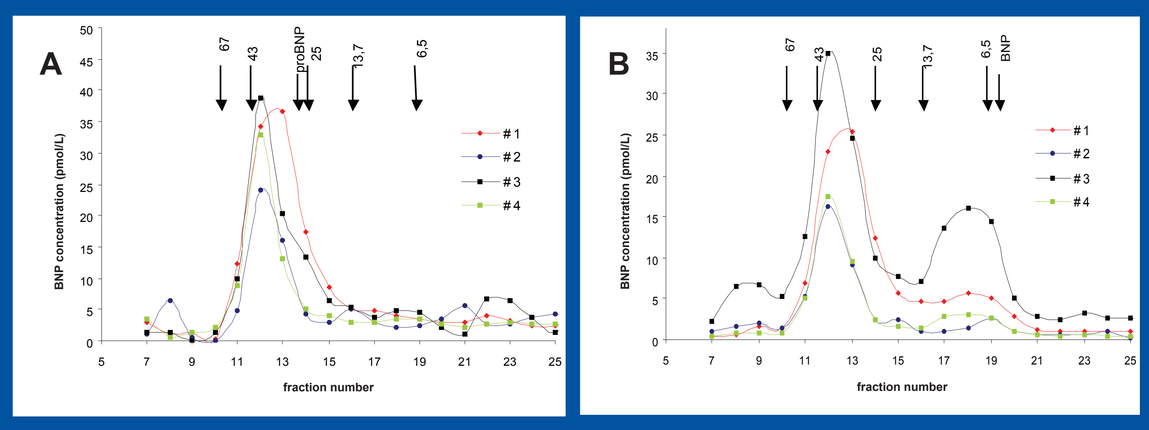

Figure 2: The immunological activities of the fractions obtained by gel filtration of four HF plasma samples (1, 2, 3 and 4) on Superdex 75 measured by A – 50E1-16F3 (proBNP assay), B – 50E1-24C5 (BNP assay). Arrows designate the position of standard proteins used as calibrators for Superdex 75 column as well as positions of recombinant proBNP and synthetic BNP. Molecular masses are indicated in kDa.

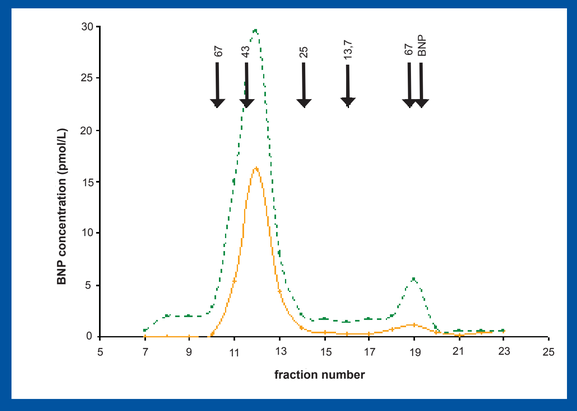

Figure 3: The immunological activity of the plasma fractions (patient #7) obtained after gel filtration on Superdex 75 as measured by 50E1-24C5 assay (—) or measured by the BNP Beckman Access (- - -). Arrows designate the position of standard proteins used as calibrators on Superdex 75 (molecular masses are indicated in kDa) as well as position of synthetic BNP.

Conclusions

On the basis of described above results, we concluded that proBNP is the major antigen form contributing to BNP-immunoreactivity in HF patients blood samples measured by both - Beckman and our in-house BNP assays.
We suppose that direct proBNP measurements by proBNP assay, utilising one antibody specific to the BNP and another to the NT-proBNP parts of the molecule, may be of the same clinical value as BNP measurements.