Immunoluminometric Assay for the Midregion of Pro-Atrial Natriuretic Peptide in Human Plasma, Nils G. Morgenthaler,* Joachim Struck, Barbara Thomas, and Andreas Bergmann (Research Department, B.R.A.H.M.S AG, Biotechnology Centre Hennigsdorf/Berlin, 16761 Hennigsdorf, Germany; * author for correspondence: fax 49-3302-883-451, e-mail n.morgenthaler@brahms.de)

The prohormone of atrial natriuretic peptide (proANP) is a polypeptide of 126 amino acids. Mature atrial natriuretic peptide (ANP) consists of amino acids 99–126 and comprises 98% of the natriuretic peptides in the circulation (1). The N-terminal portion of proANP, termed proANP1–98 or NT-proANP, has a much longer half-life than mature ANP and has therefore been suggested to be a more reliable analyte for measurement than mature ANP (2). In addition to other established indications, proANP has recently gained much interest as a potential new marker in the field of sepsis (3), emphasizing the need for a reliable assay for this molecule.

All sandwich immunoassays developed for proANP to date use an antibody against the N-terminal region of proANP1–98 combined with a second antibody against either the midregion (4) or C-terminal region (5, 6). However, under certain conditions, the N-terminal region might be minimally accessible for antibody binding (7, 8). Despite the described long half-life of proANP, results from various competitive immunoassays as well as HPLC analyses indicate that proANP1–98 can be subject to further fragmentation (9, 10).

We developed a new sandwich immunoassay for midregional proANP (amino acids 53–90; Fig. 1A). With this assay, which was named B.R.A.H.M.S SERISTRA® (B.R.A.H.M.S AG), we measured proANP values in 325 healthy controls.

EDTA-plasma samples were collected at a blood bank (Red Cross) from 325 consecutive healthy blood donors (age range, 18–67 years; 52.9% male) without clinical evidence of acute disease or a history of chronic illness. Blood donors with risk factors for heart failure were not enrolled in the study. Written consent was obtained from all donors.

For the proANP assay, tubes were coated with affinity-purified polyclonal sheep antibodies specific for amino acids 73–90 (GRGPWDSSDRSALLKS) of the molecule (Fig. 1A). Coating of the antibody was done for 20 h on polystyrene tubes (1.5 μg/tube) in 0.3 mL of buffer (10 mmol/L Tris-HCl, pH 7.8; 10 mmol/L NaCl). Tubes were blocked with 10 mmol/L sodium phosphate buffer containing 30 g/L of the polysaccharide Karion FP (Merck AG) and 5 g/L protease-free bovine serum albumin (Sigma), pH 6.8, and were lyophilized. A polyclonal sheep antibody specific for another part of proANP was used as tracer. This antibody was raised to peptide 53–72 (PEVP-PWTGEVSAPQRDGGAL) of proANP and was affinity purified on a peptide-sulfolink column. After purification, the antibody was labeled with acridinium ester as follows: 100 μg of antibody in 20 mmol/L sodium phosphate buffer, pH 8.0, was incubated for 20 min at room temperature with 10 μL of acridinium ester (1 g/L in acetonitrile; Hoechst AG). Labeled antibody was purified by HPLC with a Knauer hydroxyapatite column (buffer gradient, 1–500 mmol/L potassium phosphate, pH 6.8; flow rate, 0.8 mL/min).

Plasma proANP was measured as follows: 20 μL of patient sample (EDTA plasma) or calibrator was added in duplicate to antibody-coated tubes containing 50 mmol/L sodium phosphate buffer, pH 7.5, and incubated for 2 h at room temperature. After five washes with 2 mL of standard LUMItest© washing buffer (B.R.A.H.M.S AG), 200 μL of tracer containing acridinium ester-labeled anti-proANP antibody was added, followed by a 30-min incubation at room temperature. Tubes were washed three times with 2 mL of washing buffer, and detection was performed in a luminometer (detection time, 1 s/sample; Berthold LB952T). The relative light units reported by the chemiluminescence assay were converted to pmol/L proANP as calculated from a calibration curve that was included in every analytical run.

To prepare calibrators, proANP53–90, which contains
the two peptide moieties used for immunization of the antibodies, was chemically synthesized and diluted in horse serum (Sigma). For the highest calibrator (S6), 3000 pmol/L peptide was added to horse serum. This was diluted to prepare calibrators S1–S5, with final concentrations of 10, 30, 100, 300, and 1000 pmol/L. As controls, aliquots of horse serum containing 50 pmol/L (control I) and 500 pmol/L (control II) calibrator peptide were added at the beginning and end of each run.

The lower detection limit, determined with horse serum (mean relative light units of 20 determinations plus 2 SD), was 6.0 pmol/L. The intraassay imprecision was determined by measuring 25 human EDTA-plasma samples covering the range of the calibration curve in 10 parallel determinations. The intraassay CV was <10% for samples containing 23–3000 pmol/L proANP and <20% for samples containing 18–22.8 pmol/L. The interassay imprecision was determined similarly by measuring the same samples on 10 different days (Fig. 1B). The concentration at which the interassay CV was 20% was 18 pmol/L proANP, and the concentration at which the interassay CV was 10% was 65 pmol/L proANP. Assay accuracy was assessed by pooling five plasma samples with low proANP concentrations with five plasma samples with high proANP concentrations. Mean experimental recovery was 97% of the expected concentration. Additional recovery experiments using the calibrator peptide in two different concentrations in five samples gave a mean recovery of 106% of the calculated value. Linear dilutions (up to 1:32) were prepared from eight plasma samples. None of the samples showed a deviation >20% compared with the mean of all measurements. The assay was not influenced by albumin concentrations up to 100 g/L, bilirubin up to 4 g/L, hemoglobin up to 50 g/L, triglycerides up to 63.4 g/L, or human anti-mouse antibodies up to 822 µg/L.

Analyte stability was tested in eight different EDTA-plasma samples at 4 °C. All samples showed no difference in proANP after 48 h. Of five samples kept at 4 °C for 14 days, three showed no difference, whereas proANP in two samples was increased (>120% recovery). Four freeze–thaw cycles had no influence on the analyte concentration in eight samples (mean recovery after the first thawing, 100%; after the last thawing, 110%). Mean recovery in eight samples after 6 months of storage at −20 °C was 108%. The stability of the analyte in citrate plasma was comparable to that in EDTA plasma. However, the stability of proANP in serum or heparin plasma was influenced in a nonsystematic way; recoveries were up to 300% for a few sera. Therefore the assay is not suitable for those matrices. The influence of gel separator tubes on proANP concentrations was not studied, and the usage of those tubes is therefore not recommended.

In 325 healthy individuals, the range of proANP concentrations was 9.6–313 pmol/L. The median was 45 pmol/L (95% confidence interval, 43.0–49.1 pmol/L). The 99th percentile for the control population was 197.5 pmol/L, the 97.5th percentile was 163.9 pmol/L, the 2.5th percentile 18.4 pmol/L, and the 1st percentile was 13.6

<table>
<thead>
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<th>Age group</th>
<th>18–25 years</th>
<th>26–35 years</th>
<th>36–45 years</th>
<th>46–55 years</th>
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<td>Male (n)</td>
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<td>45</td>
<td>45.1</td>
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<td>35</td>
<td>35</td>
<td>54</td>
<td>45.1</td>
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<tr>
<td>Median, pmol/L</td>
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<td>43.3</td>
<td>45.7</td>
<td>40.0</td>
<td>41.1</td>
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<tr>
<td>Range, pmol/L</td>
<td>19.8–121.0</td>
<td>11.3–123.0</td>
<td>12.5–116.0</td>
<td>9.6–181.0</td>
<td>18.9–119.0</td>
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<tr>
<td>P, Mann–Whitney test</td>
<td>0.28</td>
<td>0.02</td>
<td>0.02</td>
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<td>0.02</td>
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</tbody>
</table>
pmol/L (defined by nonparametric percentile method). There was no significant difference in range or median proANP values between males or females (45.3 pmol/L in males; 45.0 pmol/L in females). Further stratification of the population by gender and age is shown in Table 1. Except for the 26–35 years group, there was no significant difference between males and females in the respective age groups (Mann–Whitney test). When we combined males and females in each age group, there was a significant increase of median proANP value (66.1 pmol/L) in the oldest group ($P < 0.001$ by Kruskal–Wallis ANOVA; post hoc analysis by Dunn’s multiple comparison test, $P < 0.01$ for all other groups). Spearman rank-order correlation analysis revealed a significant correlation of proANP concentration and age ($r = 0.29$; $P < 0.001$). This increase in proANP with age was also reported by earlier studies (5, 11).

We conclude that the proposed assay can measure midregional proANP in the plasma of healthy individuals. This assay may potentially be useful for evaluating proANP in patients with sepsis, systemic inflammation, or heart failure, in which proANP is reportedly increased (3, 10).

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References


In humans, the pathway for the catabolism of uracil and thymine consists of three consecutive steps. Dihydropyrimidine dehydrogenase catalyzes the reduction of uracil and thymine to 5,6-dihydrouracil and 5,6-dihydrothymine, respectively. The second step is catalyzed by dihydropyrimidinase and consists of reversible hydrolysis of 5,6-dihydrouracil and 5,6-dihydrothymine to N-carbamyl-beta-amine and N-carbamyl-alpha-amine, respectively. Finally, beta-ureidopropionase catalyzes the conversion of N-carbamyl-beta-amine and N-carbamyl-alpha-amine to beta-alanine and beta-aminoisobutyric acid, respectively, ammonia, and CO2.

Patients with a defect in one of the enzymes of the pyrimidine degradation pathway can be diagnosed by an aberrant excretion profile of the pyrimidine bases and their degradation products in urine (1). For example, in patients with a complete deficiency of dihydropyrimidinase, highly increased concentrations of 5,6-dihydrouracil and 5,6-dihydrothymine and moderately increased concentrations of uracil and thymine can be detected in urine. It has also been suggested that the 5,6-dihydrouracil/uracil ratio in plasma of patients with cancer is a prognostic indicator for the toxicity of 5-fluorouracil-based chemotherapy (2). In addition, increased concentrations of 5,6-dihydrouridine, a naturally occurring component in prokaryote and eukaryote tRNA, have been found in the urine of cancer patients.

Recently we developed a screening procedure for defects in the pyrimidine degradation pathway that combines reversed-phase HPLC with electrospray ionization tandem mass spectrometry (1). Surprisingly, the analysis of 5,6-dihydrouridine in urine with the chromatographic and MS conditions as described for the pyrimidine bases and their degradation products showed that the presence of this compound interferes with the detection of 5,6-dihydrouracil. An intense multiple-reaction monitoring signal for the transition m/z 115–73 was observed for 5,6-dihydrouridine, a transition previously selected for the detection of 5,6-dihydrouracil (see Fig. 1, A and B). In the ion source, 5,6-dihydrouridine ([M + H]+ = m/z 247) is partly degraded to m/z 115, which is identical to the [M + H]+ ion of dihydrouracil. Because 5,6-dihydrouridine coelutes with dihydrouracil, it contributes substantially to the transition peak attributed to dihydrouracil.