Radioimmunoassay of Atrial Natriuretic Polypeptide in Heat-Treated Human Plasma

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In this simple, sensitive radioimmunoassay (RIA) of atrial natriuretic peptide (hANP) in human plasma, nonspecific interference is minimized by deproteinizing the plasma by heat treatment at 85 °C for 10 min. We directly measure α-hANP in the supernates by RIA, with use of antiserum that recognizes the N-terminal region of α-hANP. The minimal detectable value was 0.4 pg per tube. The intra-assay CV was 6.6% (n = 8). The mean concentration of hANP in plasma of 54 healthy volunteers was 41 (SD 29) ng/L.

Concentrations of hANP in plasma increased after saline infusion and high salt intake for one week in patients with essential hypertension. High concentrations were also measured in patients with renal failure and congestive heart failure. This method, which requires no extraction or purification with column chromatography, is especially useful for simultaneous measurement of several samples.

Additional Keyphrases: hypertension · renal failure · heart disease · sample treatment with chromatographic cartridges compared

RIA of hANP in plasma has been widely used to investigate the physiological and pathophysiological roles of newly discovered α-hANP (1,2). To determine the concentrations of α-hANP in plasma, several investigators (2-4) have used a purification method involving Sep-Pak C18 cartridges. However, this extraction method is very complicated and time-consuming and requires a relatively large sample volume; moreover, the analytical recovery from each column is not always the same. Therefore, methods involving such column purification procedures are not suitable as routine clinical assays for use with large numbers of samples. Here we report a simple, sensitive RIA for plasma α-hANP that includes no column chromatographic steps.

Materials and Methods

Materials. We purchased synthetic α-hANP (peptides 1–28; α-hANP Met(O)12 (peptides 1–28 oxidized at position 12 of methionine); α-hANP fragments 4–28, 5–25, 5–27, 5–28, 7–23, 7–26, and 7–28; and α-hANP of rat (peptides 1–28) from the Peptide Institute, Inc., Osaka, Japan. Bovine serum albumin (BSA) and rabbit immunoglobulin G (R-IgG) were from ICN Immunobiologies, Lisle, IL 60532. Complete Freund's adjuvant was from Difco Laboratories, Detroit, MI; Na235I solution from Amersham International plc, Amersham, Bucks, U.K.; and Sep-Pak C18 cartridges from Nihon Waters Ltd., Tokyo, Japan.

α-hANP standards. We used the synthetic α-hANP (peptides 1–28) as a standard for RIA, determining a stock solution of 0.1 g/L in 0.1 mol/L acetic acid solution as 100-μL aliquots and storing them at −20 °C. We then diluted the stock solution with zero standard solution [0.2 mol of acetic acid solution, 1 g of lysozyme (EC 3.2.1.17), 1 g of NaNO3, and 1 mL of Triton X-100 per liter] to give final concentrations of α-hANP ranging from 31 to 2500 ng/L.

α-hANP-free plasma. Washed charcoal beads were packed into a 2 × 40 cm glass column, and washed again with 0.5 L of de-ionized water. We then passed 300 mL of plasma from normal subjects through the column. The first 60 mL of effluent was discarded and the subsequent fractions were pooled. We used this α-hANP-free plasma as diluent in preparing a standard curve for RIA.

Heat treatment of human plasma. We mixed 500 μL of human plasma with an equal volume of 0.1 mol/L acetic acid solution, heated the mixture at 85 °C for 10 min, and then centrifuged at 2200 × g for 10 min. We assayed 100 μL of each supernate. For the standard curve we mixed 500 μL of α-hANP-free plasma with 500 μL of each standard solution, then proceeded as with the samples.

Radioiodination and purification. We radioiodinated α-hANP by the conventional Chloramine T method (5). We purified the radioiodinated mixture by applying it to a 0.9 × 50 cm column of Sephadex G-50 (Pharmacia Japan, Tokyo, Japan), and eluting with 1 mol/L acetic acid solution. After testing the immunological activity of the material corresponding to the radioactive peaks, we pooled the fractions and stored them in 1 mol/L acetic acid solution at −20 °C until use. The specific activity of the product was approximately 1500 Ci/g. For assay, 125I-labeled α-hANP was diluted with zero standard solution.

Antiserum. We emulsified 600 μg of α-hANP conjugated with BSA in 9 mL of isotonic saline with 9 mL of complete Freund's adjuvant, then injected the mixture into six New Zealand White rabbits at four-week intervals. After six months, usable antiserum was obtained. The antiserum (ANP-2) we used in the assay was selected after evaluating titer and specificity. In the RIA, we used 100 μL of antiserum diluted 108 000-fold. Antiserum to α-hANP, for use as a control antibody, was purchased from Peninsula Co., Belmont, CA. It was diluted with phosphate buffer (50 mmol/L, pH 8.6) containing 1 g of NaNO3, 5 g of BSA, and 1 mL of Triton X-100 per liter.

RIA assay procedure. We mixed 100 μL of zero standard solution, 100 μL of heat-treated human plasma or standard solution, 100 μL of 125I-labeled α-hANP, 100 μL of the diluted antiserum, and 100 μL of R-IgG dissolved in the phosphate buffer mixture, and incubated overnight at 4 °C. Then we added 100 μL of goat antiserum to R-IgG, diluted 20-fold in the phosphate buffer mixture, and incubated at 4 °C for 2 h. After incubation, the assay mixtures were centrifuged (2200 × g, 4 °C, 30 min). The supernates were decanted, and the radioactivity of the precipitates was measured in a well-type gamma counter.

Extraction by Sep-Pak C18. We used Sep-Pak C18 cartridges treated to extract hANP from human plasma by the method of Miyata et al. (3). After evaporating the mobile phase (acetonitrile (600 mL/L) in trifluoroacetic acid solution (1 mL per liter of de-ionized water)), we dissolved the residue in 3 mL of the zero standard solution.
Blood sampling. Blood was collected into chilled tubes containing disodium EDTA (1 g/L) and aprotinin (500 int. unit/L), then centrifuged (2200 × g, 4 °C, 30 min). The resulting plasma samples were either promptly assayed or stored at -20 °C until assay.

Results

Cross reactivity of antiserum. We examined the cross reactivity of our antiserum (ANP-2) and Peninsula antibody with 10 different α-hANP fragments and α-rANP (Table 1). Removing three amino acids from the N-terminal [α-hANP (4–28)] did not significantly decrease cross reactivity, but removing four amino acids at the N-terminal [α-hANP (5–28)] significantly decreased cross reactivity. Our antiserum did not react with the fragment missing six amino acids at the N-terminal [α-hANP (7–28)]. These data (Table 1) show that our antiserum apparently recognizes only the N-terminal amino acid sequence of α-hANP, whereas the Peninsula antiserum reacts with both the N-terminal and the C-terminal amino acids.

Effect of heat treatment on α-hANP. We mixed α-hANP solutions (125, 250, 500, 1000, and 10 000 ng/L in zero standard solution) with equivalent volumes of α-hANP-free plasma. After processing these samples by heat treatment and centrifugation, we assayed hANP values in 100-μL portions of the supernates. On average, 111% of the expected value was measured, as shown in Table 2.

Chromatography of α-hANP before and after heat treatment. We added 150 ng of α-hANP to 3 mL of pooled plasma containing aprotinin (1 500 000 int. units), then applied 1 mL of this to a 1.5 × 4.5 cm column of Sephadex G-50. We eluted α-hANP with another phosphate buffer mixture (50 mmol/L, plus 9 g of NaCl, 1 g of BSA, and 500 int. units of aprotinin per liter, pH 7.5) at a flow rate of 30 mL/h, and collected 1-mL fractions. We measured the concentration of α-hANP in each fraction by RIA. The total α-hANP collected in all fractions was 57 ng; therefore, the yield of α-hANP was 117%. Another 1 mL of the pooled plasma containing 50 ng of α-hANP was mixed with 1 mL of 0.1 mol/L acetic acid solution and heated as described above. After centrifugation, we applied 1 mL of the supernate to the same column, and similarly determined the concentration of α-hANP in each fraction eluted. The total α-hANP in all fractions was 25.5 ng for a total yield of 102%. We observed no significant differences in the elution pattern and total yield of α-hANP in plasma before and after heat treatment (Figure 1).

Analytical variables. Figure 2 shows the reproducibility of the standard curve. The smallest amount of α-hANP distinguishable from zero with 95% confidence was 0.4 pg per tube. The coefficient of intra-assay variance (n = 8) was 6.6% (average for four control plasmas). Average analytical recovery of 100, 200, and 400 ng of α-hANP per liter, added to three plasma samples, was 97.4%. Twofold serial-dilutions of four samples with α-hANP-free plasma gave a good linear response to dilution.

Comparative study with Sep-Pak C18. Plasma α-hANP concentrations in 16 plasma samples were determined by both the heat-treatment method and the Sep-Pak C18 extraction method. A linear correlation was obtained between both methods. The correlation factor was 0.96 (P < 0.01), and the equation for the regression curve was y = 1.21x - 43.6.

hANP concentration in human plasma. The hANP concentration in plasma of 54 normal, seated subjects on an unrestricted sodium diet was 41 (SD 29) ng/L, ranging from 4 to 134 ng/L. Isotonic saline, infused at the rate of 1 L/h, caused a significant mean increase in plasma hANP, from 35 (SD 10) ng/L at 0 min to 52 (SD 18) ng/L at 120 min (P < 0.05) in six normal volunteers. Values also were increased in 10 patients with essential hypertension by high salt intake (20 g of NaCl each day for seven days) from 41 (SD 29) ng/L to 48 (SD 20) ng/L.

Table 1. Specificity of Antibody

<table>
<thead>
<tr>
<th>ANP peptides</th>
<th>ANP-2*</th>
<th>Peninsula</th>
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<tr>
<td>r 1-28</td>
<td>212</td>
<td>162</td>
</tr>
<tr>
<td>h 1-28</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>h 2-28</td>
<td>124</td>
<td>133</td>
</tr>
<tr>
<td>Met(O)12</td>
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<td>135</td>
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<tr>
<td>h 4-28</td>
<td>23.3</td>
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<tr>
<td>h 5-25</td>
<td>71.1</td>
<td>5.4</td>
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<tr>
<td>h 5-28</td>
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<td>156</td>
</tr>
<tr>
<td>h 7-23</td>
<td>&lt;0.2</td>
<td>&lt;0.2</td>
</tr>
<tr>
<td>h 7-26</td>
<td>0.2</td>
<td>6.1</td>
</tr>
<tr>
<td>h 7-27</td>
<td>0.7</td>
<td>152</td>
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</table>

*In-house antiserum.

Table 2. Effect of Heat Treatment on h-ANP

<table>
<thead>
<tr>
<th>ANP concn, ng/L</th>
<th>Expected</th>
<th>Observed</th>
<th>Yield, %</th>
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<tbody>
<tr>
<td>125</td>
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<tr>
<td>10 000</td>
<td>11 600</td>
<td>116</td>
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</table>

*Mean value of two determinations.

Fig. 1. Chromatograms of hANP before and after heat treatment under acidic conditions.
11) ng/L after five days of salt restriction (2 g of NaCl per day) to 81 (SD 20) ng/L (P < 0.01).

**Plasma hANP concentration in various clinical states.** We used the present method to measure hANP in plasma from patients with various disorders. Values were 63 (SD 14) ng/L (range 13–160 ng/L) in 12 patients with essential hypertension, WHO grade I-II; 150 (SD 26) ng/L (42–514 ng/L) in 27 patients with congestive heart failure due to various heart diseases; and 151 (SD 94) ng/L (12–747 ng/L) in 29 patients with chronic renal failure. Values for 10 patients with renal failure who were undergoing chronic hemodialysis were 278 (SD 253) ng/L (66–747 ng/L) and 173 (SD 153) ng/L (53–447 ng/L), before and just after hemodialysis, respectively.

### Discussion

Recently, many RIAs for hANP have been reported that are direct assays or extraction methods involving a Sep-Pak C18 column. Direct assays are believed to yield higher values because of nonspecific effects of plasma protein (6). However, Marumo et al. (7) reported a direct assay involving a sensitive and specific antibody and reasonable normal values: 32 (SD 12) ng/L (range 10–60 ng/L). Direct assays like this are, however, not considered possible when either commercially available antibody or our antibody is used. Therefore, many investigators must use the Sep-Pak C18 column method to eliminate nonspecific effects of plasma protein, a method that is very complicated and unsuited for assaying many samples concurrently. Furthermore, analytical recoveries are not consistent. Values for plasma hANP obtained by the heat-treatment method described here were equivalent to and correlated well with those obtained by the column method. Analytical recoveries of α-hANP were complete before and after the described heat treatment of plasma samples. The chromatograms of α-hANP before and after heat treatment were identical. Evidently the present method deproteinizes the plasma so that the column step can be eliminated. Use of the heat-treatment method results in reproducible values, and many samples can be assayed at the same time. Our antiserum recognizes the N-terminal region, and values for hANP in plasma of normal men by our method were in a range comparable with those previously reported by the column method. Furthermore, using our method, we observed the expected (6,9) increase in plasma hANP after saline infusion or high salt intake. These results help validate our method. The slight increase in hANP in plasma of patients with essential hypertension, the increased values in patients with congestive heart failure or renal failure, and the change in values before and after hemodialysis in patients with renal failure who were on chronic hemodialysis—all these observations are in good agreement with previous reports (10–14). These results show that the hANP in the supernate after heat treatment, as assayed with our method, is mainly α-hANP.

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### References


