Improved Radioimmunoassay of Atrial Natriuretic Peptide in Plasma
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We describe a radioimmunoassay (RIA) for measurement of atrial natriuretic peptide (ANP), based on one-step incubation and a simplified extraction procedure. The extraction was performed on a "Supelclean LC 18" column, with 2-mL plasma samples. Use of a diiodinated tracer improved the sensitivity of the RIA method. The minimal detectable value was 5 ng/L. Simplification of the extraction procedure and simultaneous incubation of the reagents provide a method more suitable for routine standard assay of ANP than those currently available. Intra- and interassay CVs were 6% (n = 12) and 11% (n = 10), respectively. The mean concentration of ANP in plasma of 32 healthy volunteers was 33 (SEM 4) ng/L. The ANP values for plasma after one-step incubation correlated well with those determined by a commercial RIA kit: r = 0.971, slope = 1.099, intercept = 1.949 ng/L (n = 25).

Additional Keyphrase: diiodinated tracer

In 1981, De Bold et al. reported a rapid, potent natriuretic and diuretic response of the rat to intravenous injection of crude myocardial atrial extracts (1). These findings have been confirmed by many other workers (2, 3). The activity was attributed to an "atrial natriuretic factor." Since these studies, a family of atrial natriuretic peptides (ANPs) has been isolated, purified, and sequenced (4). In vitro and in vivo experiments demonstrated that ANP has natriuretic and diuretic activities, relaxes smooth muscle, is stimulated by volume loading, and is antagonistic to aldosterone secretion. The only biologically active component of human ANP found in blood is a 28-residue peptide that contains a disulfide bond between cysteines 4 and 23 (5). Human atrial myocytes synthesize a pro-pro-ANP of 151 residues, which is cleaved intracellularly into pro-ANP and which is not detectable in blood (6, 7). After these early studies, synthetic ANP and anti-ANP antibodies became available, and many RIAs for ANP have been developed. Virtually all these RIAs are based on sequential incubation, and extracted plasma samples are used. Extraction of plasma seems to be a necessary step because plasma components interfere, especially at the lower concentrations of the analyte (8–10). None of the available RIAs, however, although sensitive and specific, is suitable for routine assay. We describe here a sensitive, specific, and reproducible one-step RIA, based on a simpler extraction procedure and with characteristics that fit it to routine use.

Materials and Methods

Reagents

Lyophilized synthetic ANP (Ser 99–Tyr 126), rabbit antiserum to human ANP, and the ANP RIA kit (code RIK-8798) were purchased from Peninsula Laboratories Inc., Belmont, CA. Sheep anti-rabbit antiserum and polyethylene glycol, 80 mL/L solution in phosphate-buffered saline, pH 7.2, used for bound/free separation, were supplied by Biodata S.p.A., Milan, Italy. Na¹²⁵I (100 Ci/L) in NaOH solution, pH 7–7.5, was obtained from Amersham International, Amersham, U.K. Chloramine T, sodium metabisulfite, potassium iodide, bovine serum albumin (Cohn Fraction V), gelatin, disodium EDTA, and carboxypeptidase A (EC 3.4.17.1) were supplied by Sigma Chemical Co., St. Louis, MO. Chromatography-grade methanol, acetonitrile, trifluoroacetic acid, and water, and Triton X-100 and sodium azide were purchased from Merck, Darmstadt, F.R.G. "Supelclean LC 18" columns, 3-mL tubes filled with 500 mg of silica-based monomERICally bonded packing (40-µm particles, 6-µm pores, used for ANP extraction) were obtained from Supelco Inc., Bellefonte, PA. Sep-Pak cartridges C₁₈ were from Waters/Millipore, Milford, MA.

Blood Collection, and Extraction of ANP from Plasma

We collected 5-mL blood samples into pre-cooled polystyrene tubes containing, per milliliter, 1 mg of disodium EDTA and 500 kallikrein inhibitory units of aprotinin ("Trasylol"; Bayer Pharmaceuticals Ltd., München, F.R.G.). The plasma was separated in a refrigerated centrifuge (4 °C, 1000 × g, 15 min) and stored at −70 °C until extraction. We extracted ANP by loading 2 mL of plasma onto columns pre-activated by washing with 2 mL of methanol and then with 2 mL of distilled water. The columns were equilibrated with 2.5 mL of a solution containing 50 mL of methanol and 5 mL of trifluoroacetic acid per liter of water. After the plasma samples were loaded, the columns were washed with 2 mL of a solution containing 300 mL of methanol and 5 mL of trifluoroacetic acid per liter of water. Then the ANP was eluted with 2 mL of a solution containing 5 mL of trifluoroacetic acid per liter of methanol. To the eluate we added 10 µL of a 1 mL/L solution of Triton X-100 surfactant in water. The samples were dried in a vacuum centrifuge at 4 °C. Before the extraction procedure, we added to each sample 2000 counts/min of ¹²⁵I-labeled ANP in 100 µL, to correct for extraction efficiency. The standard ¹²⁵I solution was prepared by diluting the tracer (2000 counts/min in 100 µL) to 2.5 mL in distilled water, so as to have the same volume as for the eluted samples.

RIA Method

Incubate for 24 h at 4 °C 100 µL of plasma extracts or ANP standards at concentrations of 10, 50, 20, 40, 80, 160, 320, 640, or 1280 ng/L (all in duplicate) in RIA buffer (per liter: 20 mmol of sodium phosphate dibasic, 150 mmol of NaCl, 0.1 g of thimerosal, 1.0 g of gelatin, 0.1 g of bovine serum albumin, 1 mL of Triton X-100, 500 kallikrein inhibitory kilo-units of aprotinin, pH 7.4) and 100 µL of tracer. Separate bound and free ANP by adding to each tube 100 µL of goat anti-rabbit antiserum and 1 mL of polyethylene glycol (80 mL/L solution) and centrifuge for 20 min at 2000 g.
× g at 4 °C. Measure the radioactivity with a gamma counter.

Radioiodination of Synthetic ANP

We iodinated synthetic ANP with 125I, by a modified Chloramine T method (11), by adding, in the following order, 20 μL of synthetic ANP [0.1 g/L in 60 mmol/L phosphate buffer (PB), 150 mmol/L NaCl, pH 7.4], 50 μL of PB, 10 μL of Na125I (1 mCi), and 10 μL of Chloramine T (1 g/L in PB). The reaction was stopped after 20 s by adding 20 μL of sodium sulfite (1 g/L in PB) and 100 μL of potassium iodide (200 g/L in PB). The reaction mixture was then subjected to "high performance" liquid chromatography (HPLC) on a gradient-forming chromatographic instrument (Waters Associates, Milford, MA), with a high-pore RP 304 column (250 × 10 mm column, 5-μm particle size, 33-μm pore size; Bio-Rad, Richmond, CA). The HPLC was connected to a beta-gamma-transmitting radioisotope flow detector, Model 170 (Beckman Instruments Inc., Fullerton, CA), which calculates and prints the percentage of radioactivity corresponding to each chromatographic peak. The following solutions were used as mobile phases: (A) 1 mL of trifluoroacetic acid per liter of water, and (B) 1 mL of trifluoroacetic acid per liter of acetonitrile. The mixture was eluted with 20% mobile phase B for 10 min, then for 30 min with a linear gradient up to 60% B. The flow rate was 1 mL/min. We collected 0.5-mL fractions in an automatic fraction collector (Superrack; LKB, Bromma, Sweden).

Two well-resolved peaks of radioactive ANP were eluted, identified as [125I]-mono- and [125I]-diiodotyrosyl-ANP. Their identity was confirmed by thin-layer chromatographic analysis after treatment of the eluates with carboxypeptidase A. The fractions corresponding to each peak were pooled and the acetonitrile was evaporated at 37 °C under a stream of nitrogen. A typical example is shown in Figure 1.

We then diluted the tracers to 3.75 Ci/L in RIA buffer and stored them at −70 °C. The specific activity of the tracers was determined by self-displacement (12). For all routine assays we used [125I]-diiodotyrosyl-ANP.

Results

Extraction of ANP from Disodium EDTA-Treated Human Plasma

We established the appropriate conditions for the chromatographic extraction of ANP as follows: we added 131I-labeled ANP (about 200 000 counts/min in 100 μL) to 2 mL of plasma and loaded the sample onto a pre-activated SuperCLean LC 18 column. Adsorbed ANP was eluted stepwise with 2 mL of methanol/water mixture, with the proportion of methanol being varied from 5% to 90%. In a typical experiment, 97% of input radioactivity was eluted in the range 40–90% methanol. 131I-labeled ANP eluted from the column was dried. The pellets obtained from the 40–90% methanol-eluted fractions were resuspended in 2 mL of RIA buffer and eluted by the same procedure. The elution profile of the 131I-labeled ANP was similar to that obtained in the first extraction (Figure 2). Therefore, extraction and subsequent drying of ANP did not change the adsorption/desorption behavior of this peptide on a SupelCLean LC 18 column, indicating that the ANP structure was intact.

Characteristics of the RIA Method

Incubation time. We assessed the effects of duration of incubation on the results of the assay with the diiodinated tracer. [125I]-diiodotyrosyl-ANP (14 000 counts/min per 100 μL) was incubated with 100 μL of ANP standard, 0, 10, 40, 160, or 320 ng/L in RIA buffer, and 100 μL of antiserum at 4 °C and ligand capacity determined at 12, 24, 36, and 48 h. As shown in Figure 3, equilibrium was reached by 24 h.

Analytical recovery. To evaluate the recovery of ANP from plasma samples, we added known amounts of synthetic ANP, at final concentrations of 20, 80, 160, 320, or 640 ng/L, to plasma samples obtained from a pool whose ANP concentration had been determined. ANP was extracted and determined by RIA as described. The calculated amounts were compared with the amounts found (Table 1).
Recoveries ranged from 82% to 110% (mean ± SEM = 92.4 ± 4.6%, CV 11.2%).

Specificity. The cross-reactivity of the antiserum was determined by Peninsula Laboratories. This antiserum was directed toward the C-terminal portion of the molecule.

Standard curve, detection limit, and precision. Figure 4 shows a typical standard curve. Data were processed by computer, including subtraction of nonspecific binding counts, averaging of duplicates, calculations of \((B/B_0) \times 100\) \((B_0 = \text{tracer bound at zero dose}, B = \text{tracer bound at a standard dose or unknown})\). ANP concentrations were obtained by using the spline function (concentration of the antigen plotted on the x-axis as the logarithm of the concentration). The minimum amount of ANP clearly distinguishable from zero (calculated as \(B_0 - 3 \text{ SD}\) was 5 ng/L. ANP concentrations of 378, 120, and 32 ng/L corresponded to 15%, 50%, and 85% radioligand binding relative to zero dose.

A linear correlation was observed when the values of serially diluted samples were plotted vs the diluted ratios (r = 0.997, P <0.001).

Within- and between-assay precisions were evaluated as follows. From two plasma pools, containing 27 and 140 ng of ANP per liter, 2-mL fractions were loaded onto columns (12 for each pool), eluted, vacuum-dried, resuspended in 2 mL of RIA buffer, and then assayed. The same pools were assayed in 10 consecutive assays to calculate between-

### Table 1. Analytical Recovery of ANP

<table>
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<tr>
<th>Added</th>
<th>Expected</th>
<th>Measured</th>
<th>% recovery</th>
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<tr>
<td>20</td>
<td>29</td>
<td>31.9</td>
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<td>80</td>
<td>89</td>
<td>83.7</td>
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<tr>
<td>160</td>
<td>169</td>
<td>147.0</td>
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<td>320</td>
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<td>282.9</td>
<td>86</td>
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<tr>
<td>640</td>
<td>649</td>
<td>551.6</td>
<td>85</td>
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*Original ANP concentration in the plasma pool was 9.0 ng/L, determined on 20 replicates. The relation between added (x) and measured (y) ANP was \(y = 0.84x + 13.15\) ng/L, \(r = 0.999\).
radioiodine incorporation into diiodotyrosyl-ANP was 22–24% of the total radioactivity added, with 1350–1500 Ci/g specific activity (about two iodine atoms per ANP molecule). The specific activity of [125I]monoiodotyrosyl-ANP was 460 Ci/g. Labeled material from peaks 2 and 3, corresponding to [125I]mono- and [125I]diiodotyrosyl-ANP, behaved identically with respect to antibody binding and to ANP standard displacement when used at the same weights of peptide in the reaction (data not shown), indicating that the diiodination of ANP molecule did not affect the binding capacity toward the antiserum. The percentage of binding of the tracer (14 000 counts/min in 100 μL) to the antiserum, at the dilution recommended by Peninsula, was 75–80%. We further diluted the antibody (1:2) to achieve 30–35% binding of tracer. To determine the sensitivity of the one-step RIA method with both diiodinated and monoiodinated tracer, we used 14 000 counts/min in the reaction, for both tracers, and diluted the antiserum 1:2. The minimum amount of ANP clearly distinguishable from zero (E₀ – 3 SD) with the monoiodinated tracer was 16 ng/L vs 5 ng/L with the diiodinated tracer.

Plasma ANP and Diiodinated Tracer Stability

Stability of ANP (both endogenous and exogenous) in human plasma has been studied by many investigators, with respect to different anticoagulants, different enzyme inhibitors, and different temperatures (7, 10, 13). We focused our attention on the stability of the-analyte after sample lyophilization. Fractions (2 mL) of plasma samples were lyophilized or frozen, and kept at 4 and −70 °C for 45 days. After extraction, the fractions were assayed for ANP by our RIA method. The ANP concentrations measured after the two different storage procedures correlated well (r = 0.998, P < 0.01), indicating that the lyophilization procedure did not affect ANP structure. Decreased binding (8%) and increased nonspecific binding, from 1.5% to 2.4%, were observed after the lyophilized tracer was stored at 4 °C for 45 days.

Discussion

The method described here is an improvement in the routine measurement of ANP in peripheral blood. Both extraction procedure and design of the assay have been simplified in comparison with the other methods described so far in the literature (8–10, 14–17). The use of a smaller volume of sample (2 mL of plasma rather than the 4–5 mL used by others) significantly reduces the overall extraction times, by reducing both elution and vacuum-drying time of the eluate, and considerably decreases the amount of reagents used. Replacing the more commonly used cartridges with Supelclean LC 18 columns also contributes to the simplification of this step (the columns avoid use of reservoirs). The use of smaller samples and elution volumes did not affect recovery from the standard solutions or from the biological specimens containing ANP. All the tests performed to check the recovery of ANP, in terms of percentage and of quality of recovered material, demonstrated the accuracy of the above extraction procedure.

The RIA sensitivity (5 ng/L) is similar to those of other reference methods (15, 17), despite the one-step incubation of sample, tracer, and antibody, whereas the other methods are based on a sequential analytical procedure. The good sensitivity of this one-step RIA is largely due to the use of [125I]diiodotyrosyl-ANP as tracer, which has a higher specific activity than monoiodinated tracers. The use of a one-step procedure with the same analytical performance as other methods is also a significant improvement of the RIA, reducing the overall time required. Accuracy and precision tests yielded satisfactory results. The concentrations of ANP (32 ± 4 ng/L, mean ± SEM; 9–70 ng/L, range) determined in plasma of healthy subjects overlapped the concentrations found by other investigators (9, 13, 15, 16). Furthermore, the concentrations of ANP in 25 plasma samples determined by our extraction and one-step RIA method correlated well with those determined by the recommended Peninsula procedure (r = 0.971, P < 0.001).

References