only a minor role in detecting RF by the immunoturbidimetric assay or by the latex test. Further, positivity by IgA-RF and IgG-RF ELISA in the internal control groups did not reveal any positive or borderline reactions by immunoturbidimetry or latex test for samples that were negative by IgM-ELISA.

Some have suggested (8, 9) that pregnant women have increased concentrations of RF, although others have not confirmed this (10, 11). The discrepancy is difficult to explain. The two groups who found increased concentrations of RF both used RIA. Pope et al. (9) studied sera only from the third trimester, whereas the sera examined by Meurman et al. (8) were from all stages of pregnancy but probably mainly from the first half (because the sera were originally sent for rubella antibody screening; personal communication). The three methods used in the present study did not reveal any significant changes in results for RF between early and late pregnancy. We have also carried out a follow-up study of 144 pregnancies and found no difference in prevalence of RF between pregnancy and the period four to six months after delivery (unpublished data).

In conclusion, the earlier work of Melamies et al. (1) and the present study indicate that the immunoturbidimetric assay is a good alternative to the latex test, because the correlation between the tests is good for both RA patients and non-RA subjects.

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CLIN. CHEM. 37/10, 1769–1773 (1991)

Atrial Natriuretic Peptide Assayed after Immunoextraction with Magnetic Iron Particles

Angela Torök and Botond Penke

We investigated a new method for the immunoextraction of atrial natriuretic peptide (ANP) from plasma. Anti-ANP antibody was coupled to magnetizable iron particles by various coupling methods; both carboxyl terminal and amine terminal iron particles were examined. Introducing a chemical spacer between the iron particles and the antibody improved binding with ANP. The most efficient and reliable immunoextraction involves coupling the ANP antibody to amine terminal iron particles with bis-succinimidyl-suberate. Analytical recovery of ANP from this antibody-coupled suspension was 104% (SD 7.8%). We measured extracted samples by RIA. Standards were included in the extraction step. The detection limit of the assay was 3 ng/L. Patients' samples were assayed after both immunoextraction (y) and Sep-Pak C18 extraction (x); ANP values obtained with these methods correlated highly significantly y = 1.02x - 5.57 ng/L [r = 0.9302, n = 19, SE intercept = 5.7 (not significant), SE slope = 0.10 (P < 0.0001)]. The method presented is potentially a reliable, efficacious immunoextraction method for ANP.

In the maintenance of water and salt balance, the hormone atrial natriuretic peptide (ANP) plays an important role.1 In its function as a natriuretic and vasorelaxing hormone, ANP contributes to the regulation of fluid and electrolyte balance, blood pressure, and cardiovascular homeostasis (7–5). ANP is a 28-amino-acid peptide originating from a 126-amino-acid precursor peptide (6, 7). Synthesis has been demonstrated in several tissues and organs, but the heart and the lung

1 Nonstandard abbreviations: ANP, atrial natriuretic peptide; Gla, glutaraldehyde; and DSS, bis-succinimidyl-suberate.

Institute of Medical Chemistry, Albert Szent-Györgyi Medical University, H-6720 Szeged, Dóm tér 8, Hungary.

Received February 25, 1991; accepted July 25, 1991.
are the main contributors to the concentration of ANP in plasma (8–10). The kidney is an important target organ for ANP, where the greater part of clearance takes place (2, 11–13).

ANP is used as a diagnostic and prognostic marker in various physiological disorders. Increased concentrations in plasma are caused by central volume extension or increased atrial pressure, high salt intake, or change in body posture (14–16). In pathological conditions, ANP concentrations are increased in hypervolemia, congestive heart failure, paroxysmal tachycardia, myocardial infarction, and hyperthyroidism (17–20). For measuring ANP concentrations in plasma, radioimmunoassay (RIA) is the method of choice. Owing to cross-reacting compounds and matrix effects, direct RIAs yield erroneous results (21–23). Extraction before RIA greatly enhances the specificity of the assay compared with direct measurement of ANP in plasma, although some immunoreactive material is coeluted. Therefore, an extraction step is usually included, and column extraction with Sep-Pak C18 cartridges (Waters Associates) has proven trustworthy (24). However, this extraction method has several disadvantages: some laboratory equipment and skillful laboratory technicians are required and the assay is time consuming and allows the processing of only a few samples in one run.

To circumvent several of the disadvantages of C18 column extraction, we investigated the possibility of an immunoextraction method. In contrast to other immunoextraction systems (25–27), we coupled the ANP antibody to magnetic particles.

Materials and Methods

Blood Samples

From 35 subjects attending the Clinic of Internal Medicine (St. Radboud Hospitaal, Nijmegen, The Netherlands), blood (5 mL) was collected into EDTA-containing tubes on ice. After centrifugation for 10 min at 1500 × g and 4 °C, the plasma was collected into polystyrene tubes, and 200 kallikrein inhibition units of aprotinin (Trasylol; Bayer AG, Leverkusen, F.R.G.) was added for each milliliter of plasma. The plasma samples were stored at −70 °C until analysis.

Materials

Synthetic α-hANP(99–126), α-hANP(103–126), α-hANP(105–126), α-hANP(111–126), α-ANP(104–116), α-ANP(104–123), α-ANP (99–126), ANP(1–30), and ANP(31–67) were purchased from Peninsula Laboratories Inc., U.K. Rat ANP was synthesized by solid-phase peptide synthesis at A. Szent-Györgyi Medical University, Szeged, Hungary (by Dr. B. Penke). Vasopressin, α-melanotropin, corticotropin, and α-hANP(99–126) coupled to keyhole limpet hemocyanin as antigen were obtained from Instar Corp. (Stillwater, MN).

Biomag magnetic particles were obtained from Advanced Magnetics, Inc. (Cambridge, MA), Sep-Pak C18 silica cartridges from Waters Associates (Milford, MA) and bis-succinimidyl-suberate (DSS) from Serva, F.R.G.

The reagents used for extraction were HPLC grade; all other chemicals were analytical grade.

RIA buffer consisted of, per liter, 0.2 mol of borate, 10 mmol of EDTA, 1 mL of Triton X-100, and 20 g of bovine serum albumin, pH 8.4.

The tracer used was mono-iodo-tyrosyl-ANP, which was prepared by the Protag iodination method and purified by HPLC (28).

Antibody Preparation

Immunization. Anti-ANP antiserum against α-hANP (99–126) coupled to keyhole limpet hemocyanin was raised in sheep. After a two-month period of booster injections, the titer of the antiserum was as great as 2.5 × 108. The specificity of the antiserum was characterized by measuring its cross-reactivity with several peptides (Table 1).

Antibody purification. The ANP antibody was purified before use in the coupling procedure. To a portion of antiserum we added a dropwise an equal volume of 360 g/L sodium sulfate solution. After 1 h at room temperature the mixture was centrifuged at 2000 × g and the supernate was discarded. The pellet was washed twice with 180 g/L sodium sulfate solution. Subsequently, the remaining pellet was dissolved in isotonic saline (NaCl, 9 g/L) and dialyzed against this diluent for 18 h at 4 °C. The dialyzed fraction was lyophilized and stored at 4 °C.

To remove any endogenous ANP from the antibody fraction, we dissolved the lyophilized dialysate (60 mg) in 2 mL of 0.03 mol/L HCl, precipitated the antibody with 2 mL of Polyethylene Glycol 6000, 220 g/L in 0.03 mol/L NaOH, and centrifuged. After repeating this procedure three times, we dissolved the pellet in isotonic saline and dialyzed as above.

Antibody coupling to magnetic particles. In preparing the immunoaffinity extraction system, we investigated two possibilities: antibody coupling to carboxyl terminal magnetic particles or to amine terminal magnetic particles (both Biomag brand). The coupling procedures were performed according to the manufacturer's in
struc
tions, with the activated groups being blocked by reaction with ethanolamine. For the "N-terminal" particles, coupling with glutaraldehyde (Gla method) and a modified method with DSS were also investigated (described briefly in Table 2). The antibody concentrations applied in the coupling procedure varied from 10 to 30 mg per 2 mL. The resulting antibody-coated particles were stored in 4-mL batches of wash buffer (per liter, 0.01 mol of Tris, 0.15 mol of NaCl, 1 mmol of EDTA, and 1 g of sodium azide, pH 7.4) at 4 °C.

Procedures

**ImmunoeXtraction.** Before use in immunoeXtraction, the suspension of antibody-coated particles was diluted fourfold with RIA buffer (see Materials). ANP standards in the range of 30 to 1000 ng/L were prepared by diluting synthetic α-hANP (99–126) in RIA buffer. Samples (280 μL) of plasma or standard were mixed first with 750 μL of RIA buffer, then with 200 μL of diluted particle suspension, and rotated end-over-end for 3 h at 4 °C. To separate the particle-bound fraction, we placed the sample tubes on a magnetic rack, sedimeted the particles, and discarded the supernates. We eluted ANP from the magnetic particles with 250 μL of 0.03 mol/L HCl per tube. The magnetic particles were sedimented and the supernates were collected and neutralized with an equal volume of 0.03 mol/L NaOH. The eluates were analyzed by RIA.

**C18 column extraction.** To assess the reliability of the immunoeXtraction, we simultaneously extracted several plasma samples with Sep-Pak C18 cartridges, as described earlier (29). In brief, 0.5 mL of plasma was acidified with 3 mL of 40 mL/L acetic acid. Sep-Pak C18 columns were prewashed consecutively with 5 mL of acetic acid/ethanol/water (4/86/10) by vol, 5 mL of methanol, 5 mL of distilled water, and 5 mL of acetic acid. After applying the acidified samples onto the columns, we washed each column with 3 mL of distilled water and subsequently eluted the ANP with 3 mL of the aqueous acetic acid/ethanol solution. The eluate was evaporated under a steam of nitrogen at 37 °C, and the remaining residue was dissolved in 0.5 mL of RIA buffer. Reconstituted eluates were analyzed for ANP by RIA.

**Radioimmunoassay.** For the RIA, we pipetted 100 μL of reconstituted extracts or standards into polystyrene tubes in duplicate. Antiserum (200 μL) was added and the mixture was incubated for 18 h at 4 °C. Subsequently, we added 200 μL of iodinated ANP tracer (9500 dpm) and incubated the samples for 24 h at 4 °C. To separate bound and free fractions, we used 1 mL of second-antibody precipitating solution per tube. The second antibody solution consisted of anti-sheep IgG raised in horse, 15 mL dissolved in 1 L of 0.2 mol/L borate buffer, pH 8.4, containing 2 g of bovine serum albumin and 30 g of Polyethylene Glycol 6000 per liter (28). The detection limit of the RIA was 4 pg of ANP per milliliter of plasma. Intra- and interassay coefficients of variation were 8.6% (mean 12.7 ng/L, n = 7) and 11.6% (mean 21.7 ng/L, n = 6), respectively.

**Results**

For coupling ANP antibody to iron particles we investigated three different methods. In our first experiments we found that coupling to C-terminal magnetizable particles yielded low binding of ANP tracer. We used two different methods to couple antibody to N-terminal particles: the Gla method and the DSS method, the latter being a new method for antibody coupling. Both yielded particle suspensions showing an initial binding of about 25% and both were used for further characterization.

The initial coupling experiments involved untreated antiserum. To exclude interference from endogenous ANP, we further purified the antiserum solution with pH shock and dialysis. This considerably increased the binding for both coupling methods (B_o/T = 30–40%).

The efficiency of the coupling was determined by measuring the nonspecific binding of the suspension. Repeated washing of the antibody-coupled particle suspension (pH shock) reduced the nonspecific binding from 7.5% to 4.5%.

The capacity of the antibody-coupled particles for ANP binding was determined by adding iodo-labeled ANP to serially diluted particle suspensions. Binding of the ANP tracer was 91.1% and 89.2% for the Gla-coupled particles and 94.2% and 91.7% for the DSS-coupled particles for dilutions of four- and eightfold, respectively.

Standard concentrations of ANP (30 to 1000 ng/L) were prepared in RIA buffer and in ANP-free plasma. Both series of standards were immunoeXtracted with the Gla-coupled and the DSS-coupled particles. The resulting standard curves were compared with a reference standard curve of ANP in RIA buffer measured directly.

**Table 2. Anti-ANP Antibody Coupling to "N-Terminal" Biomag Particles with Bis-succinimidyl-suberate (DSS Method)**

<table>
<thead>
<tr>
<th>Description</th>
</tr>
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<tbody>
<tr>
<td>Wash 2 mL of Biomag 4100 twice with 2 mL of coupling buffer (0.1 mol/L NaHCO3, pH 7.8), then add 3 mL of coupling buffer.</td>
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<tr>
<td>Add slowly 20 mg (50 mmol) of DSS dissolved in 0.8 mL of dioxane to vortex-mixed particle suspension and agitate for 3 h at room temperature.</td>
</tr>
<tr>
<td>Separate the magnetic particles and aspirate the supernate. Wash the particle fraction three times with an equivalent solution of dioxane and 0.1 mol/L NaHCO3.</td>
</tr>
<tr>
<td>Wash with 0.1 mol/L NaHCO3. Add 1 mL of antibody (10, 15, 20, or 30 g/L) dissolved in 0.1 mol/L NaHCO3 and shake vigorously.</td>
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<tr>
<td>Incubate the suspension overnight while rotating the samples at room temperature.</td>
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<tr>
<td>Add 2 mL of 1 mol/L ethanolamine, pH 8.7, to block remaining free activated groups; mix for 1 h with agitation. Wash again with NaHCO3.</td>
</tr>
<tr>
<td>Wash the particle fraction three times subsequently with 4 mL of 0.03 mol/L HCl and 0.03 mol/L NaOH (the last step is HCl wash) to remove weakly bound antibody. Store the final particle fraction in wash buffer (per liter: 0.01 mol of Tris, 0.15 mol of NaCl, 1 mmol of EDTA, and 1 g of sodium azide, pH 7.4).</td>
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which was used at Sep-Pak extraction. Both Gla-coupled and DSS-coupled particle suspensions yielded comparable standards curves, but were somewhat shifted from the reference curve (Figure 1). The detection limit of the immunoextraction curves was 3 ng/L.

The efficiency of the immunoextraction was determined by measuring the recovery of ANP from plasma samples. We added different amounts of ANP to a plasma pool and measured the concentrations after extraction. The optimum amount of antibody used for the DSS-coupling method was 10 g/L, which resulted in a mean analytical recovery of 104.2% (SD 7.8%, n = 5). For the Gla-coupling method the optimum amount of antibody was 15 g/L, resulting in a recovery of 85.7% (SD 7.5%, n = 5).

The effect of sample volume was determined by extracting 1- or 2-mL samples containing different concentrations of added ANP. Analytical recovery of ANP in 1-mL samples was 78.9% (SD 5.2%, n = 5), not significantly different from that in 2-mL samples (85.7% ± 7.5%, n = 5). Both of these experiments were performed with Gla-coupled N-terminal iron particles.

Parallelism of the immunoextraction assay was assessed by serial dilution of plasma samples with high ANP concentration. The results yielded \( y = 1.904x - 0.162 \) (\( r = 0.9985, \) SE intercept = 0.2967, SE slope = 0.06). The results showed a good linearity in response to dilution.

To assess the accuracy of the immunoextraction, we compared the results for patients' samples immunoeextracted with DSS-coupled particles (y) with those after Sep-Pak extraction (x). Regression of the results gave the equation \( y = 1.02x - 5.57 \) (\( r = 0.9302, n = 19, \) SE intercept = 5.70 (nonsignificant), SE slope = 0.10 (\( P < 0.0001 \)). The Gla-coupling method yielded higher concentrations: \( y = 1.68x - 18.34 \) (\( r = 0.965, n = 14, \) SE intercept = 8.74 (nonsignificant), SE slope = 0.13 (\( P < 0.0001 \)).

Discussion

Extraction of ANP is a prerequisite for accurate assay in plasma (24, 30). To enhance the efficiency and efficacy of the extraction, we developed a solid-phase immunoextraction system with ANP antibody coupled to magnetizable iron particles. Biomag magnetic iron particles are available in two forms: coated to provide carboxyl groups, "C-terminal," or to provide primary amino groups, "N-terminal."

Attempts to couple ANP antibody to C-terminal iron particles (Biomag 4125) failed, in that the capacity to bind iodinated ANP tracer was low. Coupling N-terminal iron particles (Biomag 4100) with ANP antibody was performed with two different coupling reagents: Gla and DSS. Initial binding studies revealed that both coupling methods rendered sufficient ANP binding capacity of the N-terminal antibody-coated particles. However, low zero-binding (%B/B) and high nonspecific binding indicated that either the anti-ANP serum was contaminated with high concentrations of endogenous ANP or the noncovalently-bound ANP antibody resisted binding in the coated particle suspension. Thorough washing of the antibody, applying pH shock, and dialyzing removed endogenous ANP, and a similar treatment of the coated iron particles with pH shock removed any weakly bound antibody. Any remaining free activated groups were blocked with ethanalamine. These procedures resulted in two batches of antibody-coated iron particles (Gla- and DSS-coated), which showed zero binding of about 40% and nonspecific binding of about 4%, comparable with Sep-Pak extraction data (28).

Varying the amount of ANP antibody used for the coupling reaction showed that the DSS method required less antibody (10 g/L) to achieve optimum binding results than did the Gla method (15 g/L). The resulting binding capacity was sufficient to permit the particle suspension to be diluted at least fourfold in the extraction of ANP.

Standard curves prepared after extraction with both the Gla- and the DSS-coupled particles were comparable with the directly measured ANP standard curve. Each curve was used as standard for ANP-RIA, although the %B/B values in the middle range of standard concentrations were slightly increased. The detection limit of the iron particle methods was lower than that of the direct method (3 vs 4 ng/L). Including a standard curve in the extraction series of samples will exclude water vs plasma matrix effects in the RIA. Comparing the Gla- and the DSS-coupled N-terminal iron particle suspensions indicated that, besides a greater efficiency in antibody coupling, immunoextraction with DSS-coupled iron particles showed greater recovery of ANP from plasma: 104% (SD 7.8%) vs 85.7% (SD 7.5%).

In contrast to the recovery experiment involving ANP added to plasma pools, assay of patients' samples showed a different picture. Immunoextraction with Gla-
coupled particles (y) yielded values about 50% higher than those after Sep-Pak extraction (z) \( y = 1.65x - 18.34 \), whereas immunoextraction with DSS-coupled particles \( (y') \) gave comparable values \( y' = 1.02x - 5.87 \). It is difficult to suggest plausible explanations for this phenomenon. Cross-reacting immunoactive substances remain in the eluate after Sep-Pak extraction (29). Perhaps Gla-coupled iron particles bind more cross-reacting, immunoactive ANP fragments than do DSS-coupled particles. In both coupling methods the antibodies are coupled to iron particles via a spacer molecule. Gla coupling results in a Schiff base containing two double bonds, whereas DSS coupling (active ester) results in a long spacer with free rotation. Perhaps differences in spacer configuration influence the efficiency and specificity of ANP binding. Perhaps, also, the coupling of antibodies to C-terminal iron particles failed because of the lack of a spacer molecule between antibody and iron particle.

In conclusion, we present a method for immunoextraction of ANP with magnetic iron particles coated with ANP antibody. Use of DSS-coupled antibody-coated iron particles yields assay results comparable to Sep-Pak extraction. However, the magnetic immunoextraction method has the following advantages: immunoextraction is considered more specific than other extraction systems; including plasma-based standards in the extraction step minimizes matrix effects in the RIA; use of magnetic separation increases the number of samples that can be extracted per assay; the influence of experimental conditions, e.g., oxidation of ANP, is considerably less; expensive laboratory equipment is not needed; and the antibody-coated particles might be reused. Further research is needed for characterization and optimization of the immunoextraction method.

We are indebted to Prof. Drs. Th. J. Benraad and L. M. J. W. Swinkels, University Hospital, Department of Experimental and Chemical Endocrinology, Nijmegen, The Netherlands, for their help in the laboratory work and theoretical considerations. We are grateful to P. M. M. Meulenbergh, ELTI Support, Nijmegen, for her technical assistance.

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