We have developed and validated a two-site liquid-phase immunoradiometric assay (IRMA) of atrial natriuretic peptide (ANP) in unextracted human plasma. Both radiolabeled rabbit anti-ANP IgG and polyclonal mouse anti-ANP must bind to ANP for detection, and the assay is specific for peptides with both an intact C-terminus and a disulfide bridge. The assay sensitivity (detection limit) is 0.96 pmol/L, and the working range is 2.3-300 pmol/L, with the hook effect occurring above 500 pmol/L. Results for diluted plasma from normal subjects and from patients with renal failure paralleled the standard curve; analytical recovery of ANP added to such samples averaged 94%. The between- and within-assay CVs at 8 pmol/L were 10% and 5%, respectively. The assay is sufficiently sensitive and precise to detect the postural change in ANP concentrations in normal subjects.

Numerous assays for atrial natriuretic peptide (ANP) have been described (1-6). Most involve radiolabeled antigen and a single antisera to ANP and are susceptible to interference from binding to plasma proteins and from the degrading effects of plasma peptidases, necessitating acid extraction of ANP from samples before assay.

A comparison of the results obtained by different assays demonstrated considerable lack of agreement (7). This may be attributable to different antisera specificities, with detection of ANP fragments originally present in the plasma or produced during extraction; variable recovery after extraction; and calibration differences. The extraction step is time consuming and increases imprecision; however, it improves sensitivity by concentrating the ANP.

Some ANP radioimmunoassays involving no extraction have been validated (6-11). They are suitable for measuring pathologically high ANP concentrations but are unreliable when measuring concentrations within the normal range (6), owing to lack of precision and sensitivity and the persistence of interfering effects.

Labeled antibody assays (immunoradiometric assays, IRMAS) of the sandwich type are more sensitive than labeled antigen immunoassays, have greater specificity (because two antibodies must bind for detection), and are generally quicker and easier to perform (12). They are less prone to interference from other plasma components and have been used successfully to measure other peptides in unextracted plasma (13, 14).

We describe the first liquid-phase IRMA for ANP, with use of polyclonal antisera from two different animal species. The assay is specific for active forms of ANP and is sufficiently sensitive and precise to study small changes in ANP concentrations at the lower end of the normal range without an extraction step. The assay is simple to perform and involves a short incubation time.

Materials and Methods

Materials

Reagents: Atrial natriuretic peptides alpha-h-ANP 1-28, alpha-r-ANP 1-28, beta-h-ANP 1-28, h-ANP 4-28, h-ANP 5-28, h-ANP 7-28, h-ANP 13-28, h-ANP 1-11, r-ANP 4-27, and r-ANP 5-26 (h = human, r = rat) were from Peninsula Laboratories, St. Helens, Merseyside, U.K.; ANP reference preparation 85/669 was obtained from the National Institute for Biological Standards and Controls, South Mimms, U.K.; carrier-free Na125I from Amersham International, Amersham, Bucks., U.K.; and iodogen from Pierce and Warriner Ltd., Chester, U.K. Bovine thyroglobulin, glutaraldehyde (250 mL/L aqueous solution), sodium borohydride, and pristane were from Sigma Chemical Co., Poole, U.K.; Freund's adjuvants from Difco Laboratories, Detroit, MI; and CNBr-activated Sepharose 4B and Sephacryl S-300 from Pharmacia, Milton Keynes, Bucks., U.K. Aprotinin ("Trasylol") was from Bayer Ltd., Newbury, Berks., U.K.; human albumin (45 g/L solution) from Blood Products Ltd., Braintree, U.K.; normal rabbit serum from Wellcome Reagents, Beckenham, Kent, U.K.; and sheep antimume Fc antiserum from International Laboratory Services Ltd., London, U.K. Other chemicals were from BDH Chemicals, Dagenham, Essex, U.K.

ANP-free human plasma: Pooled blood (500 mL) from healthy volunteers had been collected into 10-mL tubes containing lithium heparin; the plasma was separated by centrifugation. Endogenous ANP was allowed to decay to negligible amounts by incubating the plasma at 37 °C for 24 h (5) before adding aprotinin (500 kallikrein inhibition units per liter, kilo-KIU/L) and storing at −20 °C. Assay of this plasma gave a response indistinguishable from that of the assay diluted, typically <1% of the total radioactivity added.

Calibrants: Solutions of alpha-h-ANP 1-28 in a diluent containing, per liter, 2.5 g of human albumin, 1 mL of glacial acetic acid, 1 g of sodium azide, and 500 kilo-KIU of aprotinin were prepared at concentrations ranging from 48 to 6000 pmol/L and stored at −70 °C. Diluting these 20-fold in ANP-free plasma at the time of assay gave calibrants of 2.3-300 pmol/L. These values were confirmed in the assay by comparison with a reference preparation of ANP.

Samples: Blood was drawn from an antecubital vein between 0900 and 1200 h into a chilled syringe containing aprotinin, 500 kilo-KIU/L of blood. The plasma was separated by centrifugation (4°C, 1400 × g, 10 min) in 10-mL tubes containing lithium heparin, transferred into chilled 3-mL plastic tubes, frozen on solid CO2, and stored at −20 °C for as long as two months or at −70 °C for longer storage periods. Under these conditions, the ANP concentration in plasma has been shown to be stable (5).

Buffers: The assay diluent buffer was 50 mmol/L sodium phosphate, pH 7.4, containing 5 g of human albumin, 1 g of sodium azide, and 500 kilo-KIU of aprotinin per liter. The

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Nonstandard abbreviations: IRMA, immunoradiometric assay; ANP, atrial natriuretic peptide; KIU, kallikrein inhibition units; and PEG, polyethylene glycol.

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polyethylene glycol (PEG) buffer (100 g of PEG 6000, 5 g of sodium dodecyl sulfate, and 10 mL of Triton X-100 per liter of assay buffer) was used to aid precipitation of the bound complexes. The wash buffer was 50 mmol/L sodium phosphate, pH 7.4, containing 1 mL of Triton X-100 per liter.

Methods

**ANP conjugation and production of antibodies:** We conjugated 1 mg of ANP (alpha-h-ANP 1-28) to 2 mg of bovine thyroglobulin by using glutaraldehyde reagent (20 μL) in a total volume of 1 mL in 0.1 mol/L sodium bicarbonate. We incubated the mixture overnight, in the dark, at room temperature, cooled it to 4 °C, and added 50 μL of sodium borohydride (5 g/μL in 0.1 mol/L sodium hydroxide). After 30 min, we added a further 50 μL of sodium borohydride, incubated again for 30 min, and finally dialyzed the reaction mixture against two 1-L volumes of isotonic saline (sodium chloride, 150 mmol/L) for 24 h at 4 °C. We stored the conjugate in aliquots at −20 °C.

New Zealand White rabbits were immunized by subcutaneous injection of a 1:8 (by vol) emulsion of the aqueous conjugate (100 μg of ANP) in complete Freund’s adjuvant (800 μL). Booster immunizations used a similar emulsion made with incomplete Freund’s adjuvant. We also immunized 12-week-old Balb/c mice by intraperitoneal injection of 400 μL of the complete Freund’s emulsion (containing 50 μg of ANP) on days 1, 14, 21, and 28, together with pristane (200 μL) on day 14. After day 21, the mice developed visible ascites, from which we drew fluid weekly for up to five weeks. By this protocol, mice produced a mean of 10.75 mL (and up to 50 mL) of ascites per mouse. We assessed rabbit antiserum and mouse ascitic fluid containing polyclonal anti-ANP IgG in dilution curves, using 125I-labeled ANP, to select those of a suitably high titer.

All ascitic fluid samples collected from a single mouse were pooled for use in the IRMA. ANP-specific antibodies were not further purified because the presence of nonspecific mouse immunoglobulins is beneficial in preventing interference in two-site immunoassays (15, 16). For radiolabeling we purified ANP-specific IgG in the antiserum from a single rabbit bleeding.

**Purification and radiolabeling of rabbit anti-ANP IgG:** IgG was precipitated from 10 mL of the rabbit antiserum with sodium sulfate (180 g/L) and then affinity-purified by incubation with a suspension of 400 μg of ANP linked to 0.5 g of CNBr-activated Sepharose 4B. After 24 h, we washed the suspension with isotonic saline and packed it into a column. Low-affinity IgG was eluted with 500 mmol/L ammonium acetate/acetonitrile (80:20 by vol) at pH 7, followed by 50 mmol/L ammonium acetate/acetonitrile in the same proportions but at pH 5, 4, and 3, with immediate neutralization of the eluate with a saturated solution of sodium bicarbonate. The fraction eluted at pH 3 was collected, and its IgG concentration was calculated from its absorbance at 280 nm. We labeled 50 μg of the specific IgG with 125I, using the iodogen method (17); to separate the labeled antibody from unreacted 125I, we used a column of Sephacryl S-300, and stored the labeled antibody in aliquots at −20 °C.

For use in the assay, we added normal rabbit serum to the labeled IgG to provide a source of nonspecific rabbit immunoglobulins and to eliminate nonspecific interference in the assay (15, 16).

**Assay protocol:** The assay is performed in duplicate without extraction. Add 400 μL of freshly thawed, centrifuged sample to a tube containing 50 μL of PEG buffer. To this, add rabbit 125I-labeled anti-ANP IgG (100 000 counts/min in 50 μL of assay buffer containing normal rabbit serum, 100 mL/L), incubate the tubes at 4 °C for 4 h, and add 50 μL of anti-ANP mouse ascites (diluted 250-fold in assay buffer) and 50 μL of sheep anti-mouse Fc antiseraum (diluted 50-fold in assay buffer) to precipitate the complexes. After a further 18-h incubation at 4 °C, add 1 mL of wash buffer and separate the bound fraction by centrifugation at 4 °C for at least 30 min (1400 × g). Aspirate or decant the supernatant liquid, and count the radioactivity of the precipitate. Repeating the wash, centrifugation, and aspiration (or decanting) steps before counting the radioactivity reduces nonspecific binding from about 2% to <1%.

**Studies on the effect of posture:** From 11 healthy volunteers (five women, six men) we collected two blood samples between 0900 and 1200 h. The samples were taken from a cannula in an antecubital vein after 30 min of resting in the supine position and after 30 min of standing quietly. The cannula was flushed with 0.5 mL of heparin (1000 int. units/mL) between samples, and the first 5 mL of blood withdrawn was discarded immediately before collecting the specimen to be analyzed (10 mL).

**Results**

**Sensitivity and hook effect:** A calibration curve for the assay is shown in Figure 1. The sensitivity (minimum detectable concentration), calculated by the method of Rodbard (18) from 20 replicates of the zero concentration calibrant, was 0.96 pmol/L. The curve fitted at ANP concentrations >500 pmol/L (see Figure 3).

**Imprecision and working range:** The within-assay precision was calculated from results for 10 duplicates of each calibrant; at low ANP concentrations, precision was improved by including an additional wash step (Figure 2). The working range (CV <12%) was 2.3–300 pmol/L. Between-assay CVs, calculated from repeated assay of plasma from a normal subject and from a patient with chronic renal failure, were 10.2% (n = 11) at 8.4 pmol/L and 8.6% (n = 6) at 39 pmol/L.

**Plasma effects:** Plasma samples from four normal subjects and from three patients with renal failure were assayed undiluted and diluted two-, four-, and eightfold in ANP-free plasma. Results were parallel to the calibration

![Fig. 1. Calibration curve for the ANP IRMA, showing mean ± 2.5 SD for 10 duplicates of each standard](image-url)
curve, within the range of the assay imprecision (Table 1). Analytical recovery of ANP (4.7, 9.4, 19, and 38 pmol/L) added to plasma from two normal subjects with endogenous ANP concentrations of 4.0 and 12.6 pmol/L and of ANP (12, 24, 48, and 96 pmol/L) added to plasma from a patient with renal failure whose endogenous ANP concentration was 31 pmol/L averaged 94.1% (range 83-111%). Although high concentrations of urea, which may be found in plasma from renal failure patients, can affect antigen–antibody binding, the addition of urea, 100 mmol/L, to plasma before assay did not affect the result.

Specificity: Nine other atrial natriuretic peptides and related fragments were assayed in concentrations equimolar with those of the ANP calibrators. Because the standard curves for the different peptides were not all parallel (Figure 3), we calculated cross-reactivity by comparison with alpha-h-ANP 1-28 at 63 pmol/L (Table 2), the concentration of ANP giving 50% of maximum binding. Peptides with deletions at the N-terminal showed increased binding in the IRMA, whereas removal of even one amino acid at the C-terminal greatly reduced detection. Fragment 13-28 cross-reacted weakly. Rat ANP, which differs from human ANP only at amino acid 12, behaved almost identically to human ANP, but had slightly less maximum binding.

To determine individual antisera specificity, we compared calibration curves for the different peptides in radioimmunoassays. Peptides were incubated overnight at 4°C in assay buffer with 125I-labeled ANP as tracer and either mouse ascites (1:4000 final dilution) or rabbit antiserum (1:10 000 final dilution); this was followed by charcoal separation of bound from free label. The cross-reactivities at 50% of maximum binding are shown in Table 2. The rabbit antiserum is specific for the C-terminal of ANP, the deletion of even one amino acid at the C-terminal resulting in loss of reactivity (compare results for peptides 4-28 and 4-27). The mouse ascitic fluid antibodies recognize both the C-terminal (compare peptides 4-28 and 4-27) and the N-terminal region near amino acid 7 (compare peptides 5-28, 7-28, and 13-28), where the disulfide bond is formed; these antibodies also appear to require an intact ring because neither peptides 1-11 nor 13-28 reacted in the assay.

Effect of posture on ANP concentrations: The median concentration of ANP in plasma was 9.0 pmol/L (range 5.4–18.6) in the reclining position. This was significantly decreased (P <0.01, Wilcoxon's matched-pairs signed-ranks test) by 3.4 pmol/L (range 0.2 to 6.6) to 7.5 pmol/L (range 1.6–13.2) on standing (Figure 4).

Discussion

We have developed and validated a liquid-phase IRMA for the measurement of ANP in unextracted plasma. It is

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**Table 1. Dilution-Corrected IRMA Results for ANP (pmol/L) in Plasma from Patients with Renal Failure and Healthy Subjects after Serial Dilution**

<table>
<thead>
<tr>
<th>Dilution</th>
<th>Patients</th>
<th>Healthy Subjects</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>69</td>
<td>23</td>
</tr>
<tr>
<td>4</td>
<td>74</td>
<td>22</td>
</tr>
<tr>
<td>8</td>
<td>72</td>
<td>8.4</td>
</tr>
<tr>
<td>None</td>
<td>x2</td>
<td>x4</td>
</tr>
</tbody>
</table>

**Table 2. Cross-Reactivities of Human (h) and Rat (r) Peptides in the IRMA and in Radioimmunoassays with Mouse or Rabbit Anti-ANP Antibes**

<table>
<thead>
<tr>
<th>Peptide</th>
<th>IRMA</th>
<th>Mouse antiserum</th>
<th>Rabbit antiserum</th>
</tr>
</thead>
<tbody>
<tr>
<td>alpha-h-ANP</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>alpha-r-ANP</td>
<td>96</td>
<td>85</td>
<td>100</td>
</tr>
<tr>
<td>beta-h-ANP</td>
<td>67</td>
<td>193</td>
<td>100</td>
</tr>
<tr>
<td>h-ANP</td>
<td>198</td>
<td>100</td>
<td>125</td>
</tr>
<tr>
<td>h-ANP</td>
<td>176</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>h-ANP</td>
<td>167</td>
<td>53</td>
<td>100</td>
</tr>
<tr>
<td>h-ANP</td>
<td>2</td>
<td>&lt;5</td>
<td>48</td>
</tr>
<tr>
<td>h-ANP (1-11)</td>
<td>&lt;0.1</td>
<td>&lt;5</td>
<td>&lt;3</td>
</tr>
<tr>
<td>r-ANP</td>
<td>2</td>
<td>40</td>
<td>&lt;3</td>
</tr>
<tr>
<td>r-ANP</td>
<td>&lt;0.1</td>
<td>68</td>
<td>&lt;3</td>
</tr>
</tbody>
</table>
sufficiently sensitive and precise to detect the postural decrease in ANP concentrations in plasma of normal subjects that occurs on standing (19). This effect has been studied with assays that include extractions and is thought to be due to a reduction in atrial filling pressure on standing (20), resulting in diminished ANP secretion, but has not previously been reported for any assay not involving extraction. The recovery and dilution studies show no evidence of interference from plasma, and assay imprecision compares favorably with that of other ANP assays. In IRMAs, nonspecific binding is a major determinant of sensitivity and precision; both were improved by including an additional wash step, which reduces nonspecific binding.

Two other double-antibody sandwich assays for ANP in unextracted plasma have recently been described (21, 22). Both involved solid-phase reagents, a mixture of monoclonal and polyclonal antibodies, and either an enzyme or a radioisotope as the label. The range of values in normal subjects determined with these assays is in good agreement with the present study and with some extraction-type radioimmunoassays (2–6). The use of excess antisera in the IRMA favors rapid binding of ANP to its antibodies, at the expense of plasma proteins. The bound ANP is thereby protected against the action of peptidases, making IRMAs preferable to radioimmunoassays for measurement of ANP in unextracted plasma. We also chose an incubation temperature of 4°C to minimize ANP degradation by plasma enzymes.

The cross-reactivity studies demonstrated that the rabbit antiserum recognizes the C-terminal of ANP and that the mouse ascitic fluid antibodies also recognize part of the ring structure in the region of the disulfide bond. Neither antiserum differentiated between rat and human ANP, in which only amino acid 12 differs. These observations are consistent with the use of ANP conjugated via the N-terminal as the immunogen. The major circulating form of ANP is alpha-ANP 1-28 (23), although the possible existence of N- or C-terminal-deleted peptides in the circulation has yet to be investigated in detail. Bioassays of truncated ANP fragments demonstrated that both the disulfide bond between amino acids 7 and 23 (forming the 17-residue ring structure) and most of the C-terminal residues were required for receptor binding and biological activity (24–26). Our IRMA therefore detects the major biologically active forms of ANP because both an intact C-terminus and probably the disulfide bridge were required for the peptide to be detected. N-terminally deleted peptides had >100% cross-reactivity, possibly because of the removal of steric hindrance or conformational changes that favored increased antibody binding. Similar effects have been described with some ANP radioimmunoassays (27). In at least one bioassay (26), ANP 5-28 exhibited greater potency than did ANP 1-28; therefore, the increased reactivity in the IRMA could be appropriate to the increased biological activity. N-terminally deleted ANP fragments are found in the brain (28) but may not be significant in the circulation.

Beta-b-ANP 1-28 is a dimer of alpha-b-ANP 1-28. Equimolar amounts of the two peptides gave equivalent reactivity in the radioimmunoassay with C-terminally directed antibodies (the rabbit antiserum), suggesting that only one binding site was available on each dimer. However, beta-ANP had twice the cross-reactivity in the radioimmunoassay involving the mouse ascitic fluid, presumably because both molecules in the dimer were able to react with the more heterogeneous antibody population in the mouse ascites. Steric hindrance could be responsible for the reduced reactivity of beta-ANP in the IRMA. Whether beta-ANP circulates in plasma in significant amounts is a matter of contention (23, 29); therefore, the clinical relevance of its detection by the IRMA cannot be deduced.

The use of direct assays of defined specificity with calibration confirmed by comparison with a reference preparation, as in the present study, should result in better agreement between methods. The simplicity, specificity, small sample volume, and speed of the IRMA of ANP make it suitable for processing large numbers of samples for research and routine clinical use.

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References

Fig. 4. Effect of posture on ANP concentrations in 11 normal subjects measured with the ANP IRMA

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