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Development and Validation of a Two-Site Immunoradiometric Assay for Human Atrial Natriuretic Factor in Unextracted Plasma

Helen M. Lewis,[1] Wendy A. Ratcliffe,[2] Richard A.W. Stott,[2] Martin R. Wilkins,[1] and Peter H. Baylis[3]

This two-site immunoradiometric assay (IRMA) for human atrial natriuretic factor (ANF)99-126 in plasma utilizes a mouse monoclonal antibody raised against rat (r) ANF103-125 with specificity directed towards the ring structure of ANF, and a rabbit antiserum to human (h) ANF99-126. The monoclonal antibody is radioiodinated, and the IgG fraction of the antiserum is coated onto wells of a microtiter plate. Plasma or standard hANF99-126 (150 μL) is incubated with the radioligand in coated wells for 24 h. The detection limit is 0.9 pg per well, corresponding to 2 pmol/L, with a working range (CV <10%) from 4.5 to 540 pmol/L. Intra- and inter-assay precision are 7% and 9%, respectively, and the assay is unaffected by plasma matrix. In humans, the IRMA is specific for hANF99-126, the major circulating form of ANF, and does not cross-react with metabolites having deletions at the carboxy terminus. Plasma IRMA values in normal seated subjects were 6.6 ± 1.5 (SEM) pmol/L and results correlated with those of an extraction RIA (r = 0.81, P <0.001).

Rapid progress has been made in the study of atrial natriuretic factor (ANF), a 28-amino-acid polypeptide of cardiac origin with potent effects on natriuresis, diuresis, and vasodilatation (*1*).[4] In studies investigating the pathophysiological role of ANF, most investigators have measured the concentration of ANF in plasma by radioimmunoassay (RIA) (*2, 3*). Because most RIA methods lack sensitivity, extraction is required (e.g., by C_{18} Sep-Pak cartridges), to concentrate ANF before assay and avoid interference by unidentified plasma constituents. Such complexity limits sample throughput. Furthermore, there is wide variation in ANF concentrations in normal subjects reported by different laboratories, reflecting differences in reagents and methods, particularly assay standardization and extraction (*4*). Although direct (nonextraction) RIA methods for ANF have been developed (*5, 6*) in which selected, high-affinity antisera are used, comparison of results by extraction and direct methods reveal that the latter are less satisfactory for measurement of ANF in normal subjects (*4*). Interferences in direct methods were shown subsequently to arise from platelets and unidentified components of plasma (*7*).

Because immunometric assays offer improved assay sensitivity and specificity compared with RIA (*8*), we have developed a simple, two-site immunoradiometric assay (IRMA) for ANF in plasma. The sensitivity of the IRMA allows direct measurement of ANF in plasma from normal subjects. We describe the analytical validation of the IRMA and discuss the specificity and selection of compatible antisera.

Materials and Methods

Materials

ANF-related peptides hANF99-109, hANF111-126, hANF116-126, rANF99-126, hANF99-126, rANF103-123 (atriopeptin I), rANF103-125 (atriopeptin II), rANF103-126 (atriopeptin III), and hANF105-126 were from Peninsula Laboratories, St. Helens, Merseyside, U.K. Synthetic hANF used for clinical studies was from Shire Pharmaceuticals, Andover, Hants., U.K. [^{125}I]NaI (0.1 kCi/L), and ^{125}I-labeled rANF99-126 (2000 kCi/mol), and kits for measurement of urinary cyclic guanosine monophosphate (cyclic GMP) were purchased from Amersham International, Amersham, Bucks., U.K. Bovine serum albumin, Chloramine T, caprylic acid, aprotinin ("Trasylol"), bradykinin, angiotensin I, angiotensin II, and substance P were from Sigma Chemical Co., Poole, Dorset, U.K. Polyvinyl chloride microtiter plates (M24) were from Dynatech Laboratories, Billinghurst, Sussex, U.K. Sephacryl S200 was from Pharmacia, Milton Keynes, Bucks., U.K. Charcoal GR was from Merck, Alton, Hants., U.K. Sep-Pak C_{18} cartridges were from Waters Associates, Milford, MA.

Antiserum to hANF99-126, for RIA of ANF in plasma extracts, was from Peninsula Laboratories. Antiserum to hANF99-126 (code R13), for coating microtiter plates in the two-site assay, was raised in rabbits with hANF99-126 conjugated to bovine thyroglobulin as immunogen (*9*). Purified monoclonal antibody directed against rANF103-125 (atriopeptin II) was kindly donated by Bayer Co., Wuppertal, F.R.G., and was iodinated for use in the two-site assay. The production and characterization of this antibody have been previously described (*10*).

Methods

Plasma samples. Venous blood was drawn into chilled tubes containing lithium heparin as anticoagulant and 400 kallikrein inhibitory units (KIU) of aprotinin per milliliter of blood. The blood was centrifuged immediately at 4 °C, and the plasma was separated and stored at −20 °C. Specimens were collected from normal subjects, without a previous rest period, between 0900 and 1100 hours. Specimens with above-normal ANF concentrations were obtained from hospitalized patients with congestive cardiac failure and chronic renal failure.

In a study approved by the Ethical Committee, 10 healthy volunteers were rested for 2 h, then infused with synthetic hANF99-126 (4 pmol per kilogram body weight per minute for 60 min). Urine was collected at 30-min intervals throughout the study for measurement of cyclic GMP and urinary excretion of sodium. Blood was collected at the mid-point of each urine collection period for mea-

[1] Department of Pharmacology, University of Birmingham, U.K., B15 2TJ.

[2] Wolfson Research Laboratories, Department of Clinical Chemistry, Queen Elizabeth Medical Centre, Birmingham, U.K., B15 2TH.

[3] Endocrine Unit, Royal Victoria Infirmary, Newcastle upon Tyne, U.K., NE7 7DN.

[4] Nonstandard abbreviations: IRMA, immunoradiometric assay, ANF, atrial natriuretic factor; h, human; r, rat; and PBS, phosphate-buffered saline.

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surement of ANF in plasma.

Preparation of iodinated monoclonal antibody. Purified monoclonal antibody (20 μg) raised against rANF103-125 was iodinated in the presence of [^{125}I]NaI (0.5 mCi) and Chloramine T (25 μg), and purified by gel filtration on a column of Sephacryl S200. The specific activity of the labeled antibody was between 10 and 18 Ci/g and its shelf-life in assay diluent was six to eight weeks when stored at 4 °C.

ANF standards and controls. ANF-free plasma was prepared by mixing pooled plasma from normal subjects with activated charcoal (1 g per 20 mL of plasma) at room temperature for 2 h. Charcoal was then removed by centrifugation. Before the extraction, we supplemented the plasma with ^{125}I-labeled rANF99-126 to monitor the efficiency of the extraction step. In all cases, <5% of the added radioactivity remained in plasma after charcoal extraction.

Standards were prepared by adding known amounts of hANF99-126 to charcoal-treated plasma to give a range of assigned standard concentrations from 0.9 to 250 pg per 150 μL of plasma (2–540 pmol/L). Aliquots of these standards were stored at −70 °C until use.

Plasma pools for internal quality-control and precision studies were prepared from pooled plasma from two normal subjects, and from a patient with cardiac failure. Pools for use in measuring analytical recovery were prepared in the same way as standards by supplementing pooled plasma from healthy volunteers, plasma from patients with cardiac failure, and charcoal-treated plasma to increase the ANF concentration by 13.0, 52, and 208 pmol/L.

Assay methods. The assay buffer for all methods consisted of phosphate buffer (0.1 mol/L, pH 7.4) containing 50 mmol of NaCl, 1 g of bovine serum albumin, and 1 mL of Triton X-100 per liter.

In the RIA of extracted ANF, we acidified 5 mL of plasma with 7.5 mL of 1 mol/L acetic acid and passed this slowly through a Sep-Pak C_{18} cartridge pre-activated with 5 mL of methanol followed by 5 mL of distilled water. The ANF was eluted with 5 mL of a mixture of ethanol and 1 mol/L acetic acid (86/14, by vol). The eluate was evaporated under reduced pressure and the residue was suspended in 500 μL of assay buffer. Extraction efficiency, assessed by adding 50 nCi in 50 μL of ^{125}I-labeled rANF99-126 to 5-mL plasma samples, was 58.0% (SD 1.9%), n = 34. The extract was assayed by using a commercial antiserum to hANF99-126 and ^{125}I-labeled rANF99-126 as tracer. The limit of detection (the amount distinguishable from zero with 95% confidence) was 16.2 pmol/L (5 pg per tube); the mean intra-assay CV at 13.6 pmol/L was 9.7% (n = 8) and the mean interassay CV at 20.6 pmol/L was 18% (n = 8) (*11*).

In the two-site assay for ANF, an immunoglobulin fraction of rabbit antibody raised to hANF99-126 (Code R13), prepared by using caprylic acid, was diluted 200-fold in 0.1 mol/L sodium carbonate, pH 9.5, and dispensed into a 96-well microtiter plate (200 μL per well). This dilution of the immunoglobulin fraction gave maximum coating of the well as judged by the binding of ^{125}I-labeled rANF99-126. The plates were incubated at 4 °C for at least 16 h, then washed in phosphate-buffered saline (PBS), pH 7.4, containing, per liter, 120 mmol of sodium chloride, 2.7 mmol of potassium chloride, and 10 mmol of phosphate buffer salts. After incubation with PBS containing 1 g of bovine serum albumin per liter (200 μL per well) for 1 h at 37 °C, we washed the plates with PBS containing 0.5 mL of Tween 20 per liter, then added 150 μL of standards of patients' specimens to the antiserum-coated wells, together with 50 μL of ^{125}I-labeled rANF monoclonal antibody (150 000 counts/min). After incubation at 4 °C overnight, the contents of the wells were decanted to waste, and the plates were washed five times with the PBS–Tween 20 reagent. We cut the individual wells from the plate and counted their radioactivity for 2 min in a Model 1261 gamma counter (Pharmacia, Milton Keynes, Bucks., U.K.), using the RiaCalc program for data reduction. This analyzed two-site binding curves by spline function and calculated precision profiles, working ranges, and the minimum detection limit (based on the 95% confidence limit of the zero standard).

Cross-reaction studies. The cross-reactivities of ANF and related peptides with the anti-rANF99-125 monoclonal antibody and antiserum to hANF99-126 were determined by RIA, with use of ^{125}I-labeled rANF99-126 as tracer and a double-antibody method to separate antibody-bound and free fractions. The concentration range of the peptides studied was 3 to 100 000 ng/L. Similar studies examined the specificity of the two-site assay performed with these reagents.

Data analysis. Linear-regression analysis was applied to transformed and untransformed data, and standardized residuals were calculated. In the infusion study, changes in plasma ANF concentration or urinary cyclic GMP excretion were analyzed with respect to time and treatment by two-way analysis of variance. Significance of differences were assessed by Student's paired *t*-test.

Results

Specificity of antibodies. The specificities of antibodies with respect to ANF and related peptides are shown in Table 1. For the monoclonal antibody raised to rANF103-125, the peptides rANF99-126 and atriopeptins I–III were approximately 10-fold more potent than hANF99-126. Because rANF99-126 and hANF99-126 differ only in residue 110, this suggests that the antibody-binding site includes residue 110, located on the ring structure formed by a disulfide bond between cysteine residues 105 and 121. In contrast, the antiserum raised to hANF99-126 failed to distinguish hANF99-126 from rANF99-126, and peptides with residues deleted at both amino- and carboxy-terminals had decreased cross-reactivity. Linear peptides corresponding to amino- or carboxy-terminals of ANF failed to cross-react with both antibodies.

Characteristics of the IRMA of hANF in plasma. Figure 1 shows a typical standard curve for the direct assay of ANF in plasma. The binding with zero standard was approximately 200 counts/min, or 0.13% of the total radioligand, for the shelf life of the label; maximum binding at 540 pmol/L was typically 10 000 counts/min, or 6.6% of the total counts. The mean minimum detection limit for ANF was 0.9 pg per well, corresponding to 2 pmol/L in plasma when a 150-μL sample was assayed. The working range corresponding to a within-batch CV of <10% was 4.5 to 540 pmol/L. At ANF concentrations >540 pmol/L, specific binding decreased. A comparison of serum- and diluent-based standards yielded superimposable standard curves, indicating that specific binding was unaffected by the presence of 150 μL of plasma.

We tested the immunochemical similarity between ANF standards in plasma and endogenous ANF measured after dilution of plasma by assaying plasma from a patient with renal failure. The ANF concentration in undiluted plasma

Table 1. Cross-Reaction of ANF and Related Peptides with Individual ANF Antisera and in the Two-Site IRMA Based on These Antisera

	Cross-reaction, %		
Peptide	Monoclonal anti-rANF103-125[a]	Polyclonal anti-hANF99-126[a]	Two-site IRMA[b]
hANF99-126	100	100	100
hANF105-126	100	40	100
rANF99-126	1140	109	230
rANF103-126 (atriopeptin III)	1140	109	190
rANF103-125 (atriopeptin II)	1040	109	<1
rANF103-123 (atriopeptin I)	735	71	<1
ANF99-109	<1	<1	<1
ANF111-126	<1	<1	<1
ANF116-126	<1	<1	<1
Angiotensin I	ND	ND	<1
Angiotensin II	ND	ND	<1
Bradykinin	ND	ND	<1
Substance P	ND	ND	<1

[a] Cross-reactivities of the individual antisera with ANF-related peptides were determined by RIA with ^{125}I-labeled rANF-99-126 as radioligand, and are expressed as the mass producing 50% displacement of specific binding, relative to that of hANF99-126.

[b] Expressed as the mass corresponding to 50% of the binding of the highest standard (250 pg/tube) expressed relative to that of hANF99-126.

ND, not determined.

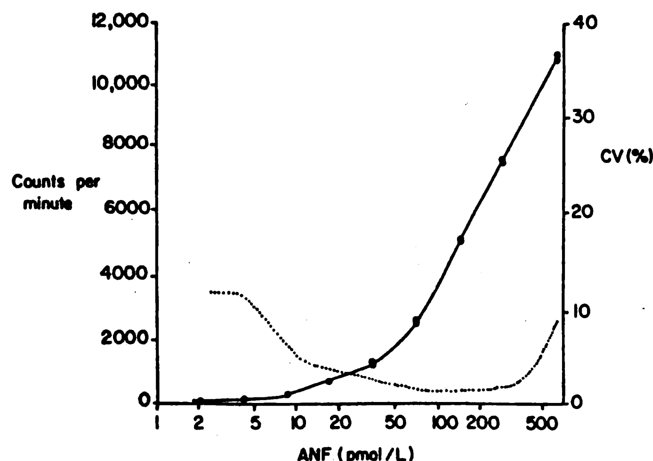

Fig. 1. Direct two-site assay for plasma ANF: standard curve and precision profile

Plasma-based ANF standards (*solid line*) were assayed in duplicate. The within-batch precision profile is indicated by the *broken line*

was 201 pmol/L; after dilution to 75%, 60%, 50%, 40%, and 25% of the original concentration by addition of charcoal-treated plasma, the concentrations measured were 145, 116, 105, 76, and 53 pmol/L, corresponding to 96%, 96%, 105%, 95%, and 105% of the expected concentrations.

The specificity of the two-site assay is shown in Table 1; rANF99-126 was approximately twice as reactive as hANF99-126 in the IRMA. Whereas deletion of the amino-terminal residues 99–104 had no significant effect on cross-reactivity, the absence of residues 124–126 decreased cross-reactivity to <1%, indicating that an intact C-terminal was required for binding.

Precision and accuracy. Intra-assay precision was studied by assaying specimens from two normal individuals 25 times, in duplicate. CVs were 7.1% and 6.8% at mean ANF concentrations of 7.9 and 12.2 pmol/L, respectively. Interassay precision was calculated from the results of quality-control specimens (pooled plasma) in 30 consecutive assays. Mean ANF concentrations were 6.5, 20.5, and 60.6 pmol/L and interassay CVs were 8.0% 9.0%, and 9.4%, respectively.

Table 2 summarizes the analytical recovery of hANF99-126 added to pooled plasma from normal subjects and to charcoal-treated plasma.

Table 2. Analytical Recovery of ANF Added to Pooled Plasma

Original ANF concn in pool, pmol/L	Added ANF concn, pmol/L: 13.0	52	208
	Measured value, as % of added[a]		
<2	119	115	88
4.9	91	101	88
25	109	90	95

[a] Values are mean of three duplicate determinations.

Normal subjects. The mean (±SEM) plasma ANF concentrations measured at 0930 hours in 10 seated normal subjects was 6.6 (1.5) pmol/L (range 2.7–17.3 pmol/L). The mean ANF concentration measured in 34 ambulatory subjects (16 women, 18 men) was 15.4 (SEM 1.4, range 3.4–28.3 pmol/L). Values for men (14.2 ± 1.6 pmol/L) were not significantly different from those for women (16.8 ± 2.3 pmol/L).

Results of extraction RIA and IRMA compared. ANF concentrations in plasma from 10 ambulatory subjects were measured by extraction RIA (x) and two-site IRMA (y). Results by the IRMA ranged from 3.4 to 28.3 pmol/L. Regression analysis yielded $y = 0.83x + 1.42$ ($r = 0.81$, $P < 0.001$). The regression gave evidence of the SD of y increasing with x; however, log transformation of y seemed to stabilize the variance: regression analysis of log y on x gave no significantly aberrant residuals.

Plasma ANF after infusion of hANF99-126. The ANF concentration in plasma from 10 subjects increased from 6.6 (SEM 1.5) pmol/L to peak at 124.8 (SEM 42) pmol/L by 15 min after the start of infusion of hANF 99-126 (Figure 2). The profile of urinary cyclic GMP excretion exactly paralleled that of immunoreactive ANF. Urinary excretion of sodium also increased substantially during the infusion period (data not shown).

Discussion

We have developed and validated a simple, two-site IRMA for direct and precise measurement in plasma of hANF99-126, the major circulating form of ANF in humans (*12*). The detection limit of the assay (2.0 pmol/L) allows direct measurement of low, physiological concentrations. The reactivity of rANF99-126 in the IRMA was about twofold that of hANF99-126, so the same methodology can be used for experimental studies in rats.

The antibodies used in the IRMA were selected from experimental evidence of their compatibility rather than from predictions based on cross-reactions of ANF-related

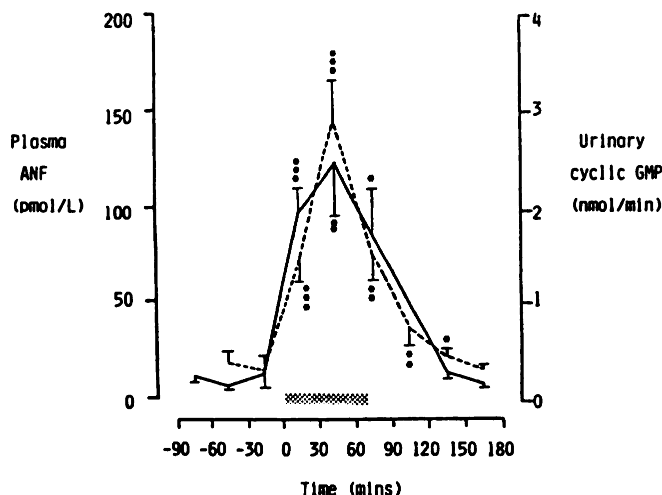

Fig. 2. Plasma ANF, as measured by two-site IRMA, and urinary cyclic GMP excretion after ANF infusion in 10 normal subjects

hANF99-126 was infused from 0 to 60 min (*shaded area*). Plasma ANF (*solid line*) and urinary cyclic GMP (*broken line*) are shown as mean (±SEM). Significant differences from baseline concentrations are shown as $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$

peptides. Because ANF has a ring structure formed by a disulfide bond between cysteine residues 105 and 121, the cross-reactivity of linear peptide fragments may not reflect that of the intact peptide. The binding site of the monoclonal antibody was located on the ring structure and included residue 110. The specificity of the rabbit antiserum was less certain, because cross-reactivity studies indicated that residues 99–104 and 124–126 were involved in antibody binding. When both antibodies were combined in the IRMA, we unexpectedly found the assay to be specific for ANF with an intact C-terminal; this may reflect either a subpopulation of antibodies specific for the C-terminal or a conformational change of ANF after antibody binding. The IRMA is therefore specific for the active form of ANF with an intact carboxy-terminus, and inactive metabolites such as ANF103-123 do not interfere in the assay.

A major factor influencing the design and performance of the IRMA was the limited quantity of rabbit antiserum available. Because this was insufficient for affinity purification and subsequent radiolabeling, we used the antibody raised in rabbit as the capture antibody, even though it was of higher affinity than the monoclonal. Had it been possible to use the polyclonal antibody as radiolabel, we might have gained further assay sensitivity, given that a lower detection limit is generally obtained by labeling the higher-affinity antibody (*13*). In addition, perhaps the high-dose "hook" effect in the IRMA could have been avoided by using the higher binding capacity of the monoclonal antibody in coating the wells. Recently Hashida et al. (*14*) described a two-site enzyme immunoassay of high sensitivity (30 fg per tube), in which a monoclonal antibody to hANF99-126 was used as the capture antibody and rabbit Fab′ conjugated to horseradish peroxidase was the label. The specificities of these antibodies were similar to those described here except that cross-reaction with rANF99-126 was low. These two studies confirm the feasibility of developing two-site assays for ANF. The high sensitivity of such assays exceeds that currently achieved by most radioreceptor assays (*15*) and RIA methods (*7, 11*). A disadvantage is the large quantity of antiserum or monoclonal antibody required; currently, these are in short supply or, if commercially available, are prohibitively expensive.

The analytical performance of the IRMA compares favorably with those of extraction and direct RIA methods. We detected no interference by plasma matrix, whereas direct RIA methods are affected by plasma components (*7*). Analytical recovery was approximately quantitative. Although the working range for ANF was restricted by a high-dose "hook" effect for concentrations exceeding 540 pmol/L, such concentrations are seldom seen in pathological situations (*16, 17*). The detection limit of the IRMA (2 pmol/L) was satisfactory for clinical studies: all normal, rested subjects had detectable ANF concentrations (mean 6.6 pmol/L), in agreement with those found by the two-site enzyme immunoassay (mean 7.9 pmol/L). As expected, plasma concentrations of ANF were higher in ambulatory than in seated subjects (*18*). The close correlation of ANF concentrations in plasma with the concentrations of urinary cyclic GMP during infusion of hANF99-126 suggests that measured immunoreactivity reflects biological activity (*19, 20*).

In conclusion, the two-site IRMA offers significant advantages over conventional extraction/RIA methods in terms of sample volume, technical simplicity, and precision. It is well suited for clinical applications, particularly when accurate measurements of biologically active peptide are required.

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References

1. Anderson JV, Bloom SR. Atrial natriuretic peptide: what is the excitement all about? J Endocrinol 1986;110:7–17.
2. Sagnella GA, Nolan DA, Shore AC, MacGregor GA. Effects of synthetic atrial natriuretic peptides on sodium–potassium transport in human erythrocytes. Clin Sci 1985;69:223–6.
3. Gutkowska J, Bourassa M, Roy D, et al. Immunoreactive atrial natriuretic factor (IR-ANF) in human plasma. Biochem Biophys Res Commun 1985;128:1350–7.
4. Dzau VJ, Robertson JIS, Richards AM, Gaines Das RE, Poole S. International collaborative study of the proposed international standard for atrial natriuretic factor: an initial analysis of radioimmunoassay results. J Hypertension 1987;5(Suppl 5):S27–8.
5. Chang JK, Chang D, Song DL, Ji ZS, Tang J. Immunohistochemical and radioimmunoassay of atrial natriuretic polypeptide in the heart and plasma of rats and humans. Fed Proc Fed Am Soc Exp Biol 1985;44:1241.
6. Tikkanen I, Fyhrquist F, Metsarinne K, Leidenius R. Plasma atrial natriuretic peptide in cardiac disease and during infusion in healthy volunteers. Lancet 1985;ii:66–9.
7. Richards AM, Tonolo G, McIntyre GD, Leckie BJ, Robertson JIS. Radio-immunoassay for plasma alpha human atrial natriuretic peptide; a comparison of direct and preextracted methods. J Hypertension 1987;5:227–36.
8. Ekins RP. Towards immunoassays of greater sensitivity, specificity, and speed; an overview. In: Albertini A, Ekins R, eds. Monoclonal antibodies and developments in immunoassay. Amsterdam: Elsevier/North Holland Biomedical Press, 1981:3–21.
9. Charlton J, Mbanya J-C, Thomas TH, Baylis PH. Radioimmunoassay of human atrial natriuretic peptide: assessment of two antisera. Clin Sci 1987;75(Suppl 17):13p–4p.
10. John A, Stasch J-P, Neuser D, Hirth C, Morich FJ. The use of a monoclonal antibody to measure plasma atriopeptins in rats. Life Sci 1986;38:1991–7.
11. Wilkins MR, Wood JA, Adu D, Lote CJ, Kendall MJ. Change in plasma immunoreactive atrial natriuretic peptide during sequential ultrafiltration and haemodialysis. Clin Sci 1986;71:157–60.

12. Schwartz D, Geller DM, Manning PT, et al. Ser-Leu-Arg-Arg-atriopeptin III: the major circulating form of atrial peptide. Science 1985;229:397–400.
13. Hunter WM, Bennie JG, Budd PS, et al. Immunoradiometric assays using monoclonal antibodies. In: Hunter WM, Corrie JET, eds. Immunoassays for clinical chemistry. Edinburgh: Churchill Livingstone, 1983:531–44.
14. Hashida S, Ishikawa E, Nakao K, Mukoyama M, Imura H. Enzyme immunoassay for α-human atrial natriuretic polypeptide—direct measurement of plasma level. Clin Chim Acta 1988;175:11–8.
15. Bürgisser E, Raine AEG, Erne P, Kamber B, Bühler FR. Human cardiac plasma concentrations of atrial natriuretic peptide quantified by radioreceptor assay. Biochem Biophys Res Commun 1985;133:1201–9.
16. Shenker Y, Port FK, Swatz RD, Gross MD, Grekin RJ. Atrial natriuretic hormone secretion in patients with renal failure. Life Sci 1987;41:1635–44.
17. Cody RJ, Atlas SA, Laragh JH, et al. Atrial natriuretic factor in normal subjects and heart failure patients. J Clin Invest 1986;78:1362–74.
18. Cuneo RC, Espiner EA, Nicholls MG, Yandle TG. Exercise-induced increase in plasma atrial natriuretic peptide and effect of sodium loading in normal man. Horm Metab Res 1988;20:115–7.
19. Hamet P, Tremblay J, Pang SC, et al. Cyclic GMP as mediator and biological marker of atrial natriuretic factor. J Hypertension 1986;4:S49–56.
20. Huang C-L, Ives HE, Cogan MG. In vivo evidence that cGMP is the second messenger for atrial natriuretic factor. Proc Natl Acad Sci USA 1986;83:8015–8.