



Analytical performance and clinical usefulness of a commercially available IRMA kit for measuring atrial natriuretic peptide in patients with heart failure

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We evaluated the analytical characteristics and clinical usefulness of a commercially available IRMA kit for measuring plasma concentrations of atrial natriuretic peptide (ANP) in healthy subjects and in patients with heart failure. The method uses two monoclonal antibodies prepared against sterically remote epitopes of the ANP molecule; the first antibody is coated on the solid-phase beads, and the second is radiolabeled with ^{125}I . Fifty-nine healthy subjects and 77 patients with heart failure were studied. After subjects had rested 20 min in a recumbent position, blood samples were collected from a brachial vein into ice-chilled disposable polypropylene tubes containing aprotinin and EDTA. Plasma samples were immediately separated by centrifugation and stored at -20°C until assay. The working range (CV $<15\%$) was 10–2000 ng/L. The detection limit (2.13 ± 0.91 ng/L) was similar to those reported for other IRMAs but was much better than those of RIAs. For healthy subjects, the results of this method (18.0 ± 10.6 ng/L, range 4.7–63 ng/L, median 16.7 ng/L, $n = 59$) were similar to those generally reported for the most accurate methods, i.e., those using preliminary extraction and chromatographic purification of plasma samples. Measured plasma ANP was significantly associated with the severity of clinical symptoms, i.e., NYHA class (ANOVA, $P < 0.0001$), and with the left ventricular ejection fraction ($n = 62$, $r = 0.618$, $P < 0.0001$). Patients with severe heart failure showed greatly increased values (NYHA III–IV: 257.4 ± 196.6 ng/L, $n = 23$).

INDEXING TERMS: natriuretic peptides • sample handling • cardiac diseases

The secretory granules of mammalian atrial cardiocytes contain a hormonal factor with natriuretic and arterial smooth muscle relaxant activities [1–3]. The cardiac peptide, termed atrial natriuretic peptide (ANP), has been purified, and the amino sequences of several forms of ANP have been determined; the biologically active component of human ANP was identified as a 28-residue peptide (ahANP_{99–126}) of the 126-residue propeptide (pro-ANP) [2, 3].

Plasma ANP is markedly increased in diseases characterized by an expanded fluid volume, including cardiac and renal insufficiency [2–4]. Circulating ANP increases with the progression of cardiac insufficiency and with the deterioration of hemodynamics; mortality and ANP are positively correlated in severe heart failure [5–7].

ANP commonly is measured by RIA, but these methods present several analytical problems [8, 9], mainly with detection limits and accuracy. An ideal assay of ANP should detect only the biologically active peptide (ahANP_{99–126}) and not its precursors (pro-ANP_{1–126}, γ ANP) or metabolites (e.g., ANP_{124–126}, ANP_{107–126}, ANP_{112–126}, and ANP_{99–105/106–126}, generally called “cleaved” ANP) or other family-related peptides (brain natriuretic peptide, C natriuretic peptide, and urodilatin) [2–4].

More recently, IRMA methods for measuring plasma ANP have been reported [10, 11] and currently show better sensitivity, precision, and accuracy than the previously reported RIAs. In addition, because IRMAs do not generally require preliminary extraction or purification of the plasma sample, they may be more suitable than RIAs for the routine assay of plasma ANP concentrations.

Given the lack of data concerning the usefulness of IRMA methods in follow-up of patients with cardiac diseases, we evaluated the analytical characteristics and the clinical utility of a commercially available IRMA kit for assaying plasma ANP.

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Materials and Methods

EXPERIMENTAL SUBJECTS

We studied 59 subjects (ages 20–75 years, 25 men and 34 women), who denied serious disease (past or present) and the use of any drug for at least 3 weeks before the study. All had normal results for common chemical and hematological tests.

We also enrolled 77 cardiac patients (ages 20–75 years, 47 men and 30 women) admitted for either coronary artery disease (~70%) or idiopathic dilated cardiomyopathy. All patients underwent a thorough clinical history and physical examination and two-dimensional color Doppler echocardiography; we also assessed myocardial contractility, dimensions, and function by radionuclide ventriculography or hemodynamic study (or both), except in 15 patients with no or only very mild symptoms of heart failure (New York Heart Association functional stage I: NYHA I). We stopped administration of all drugs at least 3 days before the study except for patients with overt congestive heart failure. Patients with atrial fibrillation or other arrhythmias, which can affect the secretion and metabolism of ANP, were excluded from the study.

Informed consent was obtained from all subjects studied, and the protocol study was approved by the local ethics commission.

PLASMA SAMPLES

Unknown plasma samples. Unless otherwise stated, blood was collected from a brachial vein after at least 20 min of rest in recumbent position between 0800 and 1000 after an overnight fast. Moreover, in some cardiac patients, to evaluate the influence of body posture on ANP concentrations, we studied blood samples that had been collected immediately, and (or) after 20 min of rest in recumbent position, and (or) after 20 min of upright position. Finally, in some cardiac patients, who underwent complete hemodynamic studies because of their cardiac disease, blood samples were also taken from different sites (e.g., brachial vein, aorta, pulmonary artery, and inferior vena cava).

Immediately after collection, the blood samples were put into ice-chilled disposable polypropylene tubes containing aprotinin (500 000 kIU/L of plasma) and EDTA (1 g/L of plasma). Plasma samples were separated without delay by centrifugation for 10 min at 4 °C, and then frozen and stored at –20 °C in different 1-mL aliquots in polypropylene assay tubes until assay (generally within 2 weeks).

Plasma pools. Four plasma pools were prepared for use in quality-control studies. Two quality-control pools were prepared from plasma samples collected (as described above) from healthy subjects; two other pools of plasma were collected from patients with heart failure. Aliquots (1 mL) of ~30 different plasma samples from healthy subjects or cardiac patients were collected in two separate beakers. After a 30-min agitation at room temperature with magnetic stirring, 1-mL aliquots from each pool were put into separate polypropylene tubes and stored at –20 °C until assay.

ANP-free pooled plasma was prepared by treating pooled plasma from healthy subjects with grossly ground charcoal (250 g/L of plasma), as previously described [12].

ANP stability in blood or plasma. Assuming that ¹²⁵I-labeled ANP is degraded by the same enzymes [mainly endopeptidase (EC 3.4.24.11), also called enkephalinase and atriopeptidase] [2–4] and in the same way as the native circulating peptide hormone [13–15], we added known amounts of freshly prepared ¹²⁵I-labeled ANP to some blood or plasma specimens to investigate the ANP stability. Labeled ANP was prepared as previously described in detail [13]. Briefly, synthetic α hANP_{99–126} (supplied by Bachem Feinchemikalien, Bubendorf, Switzerland, or by Calbiochem–Novabiochem, Lauffelfingen, Switzerland) was iodinated with Na¹²⁵I (Sorin, Saluggia Vercelli, Italy), and the mixture was then purified by both ion-exchange chromatography and HPLC [13]. Degradation of ¹²⁵I-labeled ANP was then determined by a chromatographic procedure, as previously described [13–17].

ANP ASSAY

Plasma ANP was measured (in at least duplicate) with a direct (nonextraction) IRMA kit (Shionoria ANP; manufactured by Shionogi Co., and distributed by CIS Bio International, Gif-sur-Yvette, France). This method, a solid-phase sandwich IRMA, uses two monoclonal antibodies prepared against two sterically remote epitopes of ANP molecule. The first antibody is coated onto beads (solid phase), and the second is radiolabeled with ¹²⁵I.

Briefly, the assay protocol, as suggested by the manufacturer, is as follows. In polypropylene assay tubes combine 100 μ L of calibrator solutions (containing 5, 10, 20, 60, 200, 600, and 2000 ng/L α hANP_{99–126}), 200 μ L of buffer, and 1 bead (coated with the first monoclonal anti-ANP antibody) and gently vortex-mix. After incubating all samples for 3 h at room temperature (18–22 °C, first incubation), aspirate the contents of the tubes as completely as possible, wash the tubes twice with 1 mL of washing solution (provided in the kit), and add 300 μ L of solution containing the labeled monoclonal anti-ANP antibody (~150 000–200 000 counts/min) to each tube. Gently vortex-mix, and incubate all samples for 14–20 h at 4–8 °C (second incubation). Again aspirate the contents of the tubes and wash twice with 1 mL of the washing solution. Although the manufacturer does not specify this point, it is important to perform the aspiration/washing steps at low temperature, i.e., keep the tubes in an ice bath to not perturb the equilibrium of antigen/antibody complex reached during this step. Finally, count for 1 min with a gamma scintillation counter the radioactivity remaining bound to the beads.

As indicated by the manufacturer, the IRMA shows cross-reactions of 118% with metabolite α hANP_{106–126}, 110% with metabolite α hANP_{104–126}, and 105% with β hANP. In contrast, other NH₂-terminal (such as α hANP_{99–109}) or COOH-terminal (such as α hANP_{112–126}, α hANP_{117–126}) metabolites and peptide hormones (including brain natriuretic peptide, endothelins, corticotropin, angiotensins, vasopressin, osteocalcin) do not cross-react significantly (all $\leq 0.01\%$). These data indicate that this IRMA is specific for the biologically active part of the peptide [1–4].

STATISTICAL ANALYSIS

All sample values and the other data for quality control (including the sensitivity and the imprecision profile) were calculated for each assay by a previously described computer program [18]. The interpolation of the dose-response curves was computed with a four-parameter logistic function [18]. In particular, the detection limit of the method was defined as the minimum amount of hormone distinguishable from zero (mean zero value + 2 SD). The working range was arbitrarily defined as the concentration range of ANP measured with an imprecision profile (CV) <15%.

The statistical analysis was carried out by using a Macintosh IIsi personal computer and the Stat-View 4.0 and Super-ANOVA programs (Abacus Concepts, Berkeley, CA). For comparisons between two paired groups, the paired *t*-test was used; for unpaired comparisons, the unpaired *t*-test or the nonparametric Mann-Whitney *U*-test was used (the nonparametric test was used when the variances of the two groups were significantly different by the *F*-test). Moreover, the data for more than two independent groups (of patients or controls) were analyzed by ANOVA, and the significance of differences between the pairs of means was tested by Scheffé's test, with logarithmic transformation of data when necessary. Scheffé's test was chosen for multiple comparisons because it is considered one of the most conservative tests and because it is very robust to violations of the assumptions typically associated with multiple comparison procedures, including heterogeneous variances.

The results are expressed as mean \pm SD in the text and as mean \pm SE in Fig. 1.

Results

STABILITY OF ANP IN BLOOD AND PLASMA

A known amount of labeled ANP was added to six blood samples containing plasma protease inhibitors (i.e., EDTA and aprotinin) and collected from the healthy control subjects or from cardiac patients. These specimens were then incubated for 5 min at room temperature. After separation by centrifugation, the percentage of ANP degradation in plasma was tested by HPLC (mean \pm SD degradation, 9.7% \pm 1.7%). Moreover, another aliquot of two of these blood specimens was incubated for 2 h at room temperature before centrifugation; the mean degradation found in plasma after separation by centrifugation averaged 33.0%. Finally, another aliquot of two of these blood specimens was hemolyzed by freezing at -80°C for 10 min and then incubated at room temperature for 5 min before centrifugation; the percentage of degradation in these two specimens averaged 56.5%.

A known amount of ^{125}I -labeled ANP was added to four 2-mL specimens from the same plasma pool, with or without plasma protease inhibitors (EDTA and aprotinin); two of these samples were incubated for 2 min at 37°C and the others for 30 min at 37°C . After incubation, the percentage of degradation was tested in duplicate by HPLC assay. The degradation of tracer in the specimens incubated for 2 min averaged 4.2% for the specimen not containing protease inhibitors, whereas results for the specimen containing inhibitors were superimposable on that of the tracer added (i.e., within 0.8%). After an incubation

of 30 min at 37°C , the specimen that did not contain protease inhibitors showed 70.2% degradation; the samples containing inhibitors lost only 3.7% of their initial value.

Moreover, the ANP concentrations measured by the IRMA in the four plasma control pools, prepared with plasma samples collected from the control subjects or the patients with heart failure, were shown to be stable for 12 months (see *Between-assay precision*).

EVALUATION OF IRMA ANALYTICAL PERFORMANCE

Calibration curve, working range, and sensitivity. Fig. 1 reports the comparison between the calibration curve and the related imprecision profile of the IRMA obtained by performing the first incubation of the assay as recommended by the manufacturer (3 h at room temperature, 18 – 22°C) and the calibration curve and imprecision profile obtained by using a longer first incubation at a lower temperature (12 h at 4 – 8°C). As these data indicate, we were able to increase the detection limit of the IRMA kit by changing the time and temperature of the first incubation. Indeed, when the IRMA kit is used according to the manufacturer's instructions, ANP concentrations from ~ 25 to 2000 ng/L can be considered as the working range of the assay (i.e., the range of ANP concentrations that can be measured with an imprecision of <15%; Fig. 1B). Using a longer incubation time at lower temperature, however, yielded a wider working range (from ~ 10 to 2000 ng/L). Moreover, the mean computed detection limit [18] of the IRMA obtained by the manufacturer's instructions was 5.7 ± 2.3 ng/L ($n = 17$), significantly higher than the detection limit (2.2 ± 0.9 ng/L, $n = 13$) obtained by using a first incubation at 4°C for 12 h ($P < 0.0001$, Mann-Whitney *U*-test).

Between-assay precision. The between-assay precision was repeatedly tested throughout 1 year by assaying four plasma pools with different ANP concentrations. The observed values were 11.4% (mean \pm SD = 22.6 ± 2.6 ng/L, $n = 12$) and 10.7% (mean \pm SD = 25.6 ± 2.7 ng/L, $n = 8$) for the two pools collected from the control subjects, and 8.0% (178.6 ± 14.3 ng/L, $n = 12$) and 6.7% (162.2 ± 10.8 ng/L, $n = 8$) for the two pools collected from patients with cardiac insufficiency.

Dilution test. As assayed with the IRMA, various volumes (from 50 to 300 μL) of plasma samples of two patients with heart failure, containing relatively high concentrations of ANP, showed a close linear regression between the plasma volume assayed and the ANP measured (Fig. 2). Although the manufacturer of IRMA kit suggests assaying 100 μL of a patient's plasma, the results of these dilution tests indicate that one can validly increase the volume (until 300 μL) of plasma assayed and thus improve the precision of the determination of samples with low ANP contents, as indicated in Fig. 1B.

Recovery test. An ampoule containing 100 μg of synthetic ANP (Calbiochem-Novabiochem) was appropriately diluted with the IRMA kit buffer and then repeatedly ($n = 8$) assayed; the mean analytical recovery was $98.8\% \pm 5.3\%$. Furthermore, the mean recovery of known amounts (5–2000 ng/L) of synthetic peptide

added to seven ANP-free plasma samples (containing EDTA and aprotinin) was $99.5\% \pm 18.8\%$.

CLINICAL RESULTS

Body posture and ANP concentrations. Blood samples were collected from 16 cardiac patients (NYHA functional class I to III) immediately after the assumption of the recumbent position and then at rest after 20 min of recumbent position. The mean ANP value measured after 20 min of recumbent rest (49.7 ± 41.9

ng/L) was significantly ($P = 0.0477$, paired t -test) greater than that collected immediately after the assumption of the recumbent position (43.6 ± 38.8 ng/L). Moreover, in 8 other cardiac patients (NYHA I to III), blood samples collected after 10 and 20 min of recumbent position showed no significant differences between the mean ANP values measured: 91.4 ± 78.1 ng/L and 76.8 ± 72.3 ng/L, respectively. Finally, in 8 cardiac patients with no or only very mild symptoms of heart failure (NYHA I), blood samples were collected after 20 min of recumbent rest and also after 20 min in the upright position. The mean ANP value found in the recumbent position (35.3 ± 29.9 ng/L) was significantly higher ($P = 0.0330$, paired t -test) than that observed in the upright position (28.6 ± 24.0 ng/L).

ANP concentrations in control subjects. The ANP concentrations obtained in 59 healthy adults of both sexes were 18.0 ± 10.6 ng/L (range 4.7–63 ng/L, median 16.7 ng/L), a value very similar to that suggested by the manufacturer (19.0 ± 12.0 ng/L). Moreover, there was no difference between the mean value in men (18.6 ± 14.1 ng/L) and in women (17.6 ± 8.0 ng/L).

ANP concentrations in cardiac patients. Plasma ANP was also measured in 77 patients with cardiac disease at different stages of heart failure; as a whole, ANP concentrations in the cardiac patients (119.5 ± 150.7 ng/L) were significantly ($P < 0.0001$, Mann-Whitney U -test) higher than those in the control subjects (18.0 ± 10.6 ng/L). However, because ANP circulating concentrations tend to increase with the progression of clinical severity of the disease, some patients with only mild ventricular dysfunction may show ANP concentrations within the normal reference range (NYHA I, 43.2 ± 35.0 ng/L; NYHA II, 79.8 ± 90.1

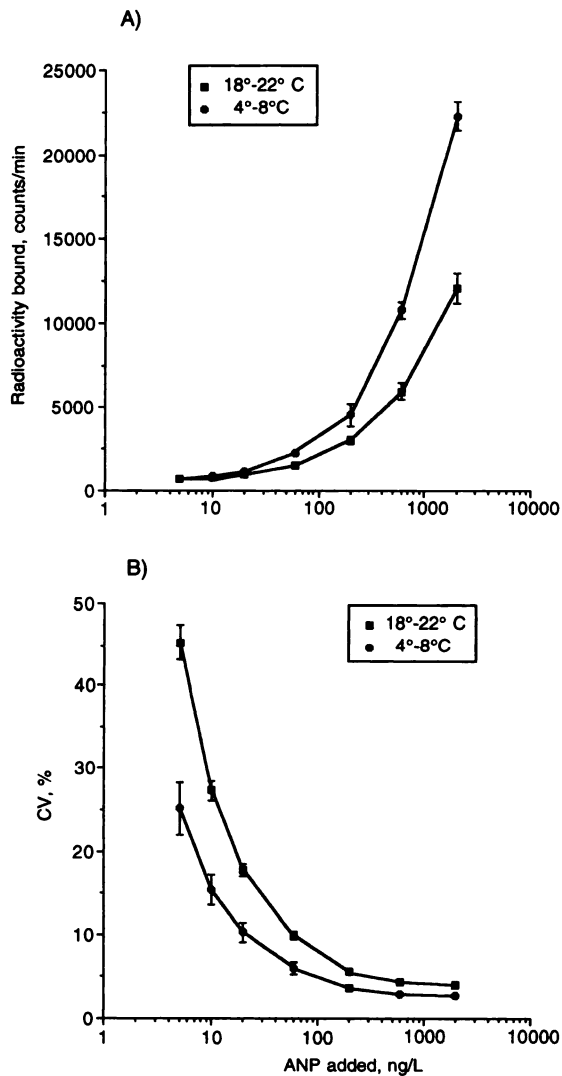


Fig. 1. (A) Mean (\pm SE, $n = 12$) calibration curve of the IRMA kit, obtained by performing the first incubation according to the manufacturer's instructions (at room temperature, $18\text{--}22^\circ\text{C}$ for 3 h), vs the mean (\pm SE, $n = 8$) calibration curve obtained by performing the first incubation at $4\text{--}8^\circ\text{C}$ for 12 h; (B) mean (\pm SE) imprecision profiles (CV, %) of the IRMA kit, obtained by performing the first incubation by the manufacturer's instructions, vs that found by using a first incubation at $4\text{--}8^\circ\text{C}$ for 12 h.

The imprecision profile was calculated from results for all of the samples measured in one assay (i.e., calibrators, quality-control samples, and unknown samples from healthy subjects or patients). The working range was arbitrarily defined as the range of ANP concentration measured with an imprecision of $<15\%$.

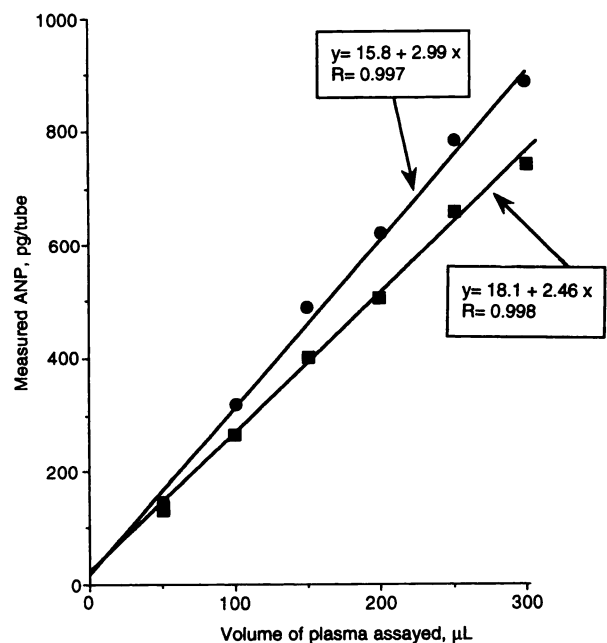


Fig. 2. Dilution test: effect of assaying different volumes ($50\text{--}300\ \mu\text{L}$) of plasma samples of two patients with heart failure, containing relatively high concentrations of ANP, with the IRMA kit.

ng/L), whereas cardiac patients with more severe symptoms of heart failure generally show greatly increased hormonal values (NYHA III and IV, 257.4 ± 196.6 ng/L) (Fig. 3).

Moreover, we observed a significant negative linear regression ($P < 0.0001$) between the logarithmic transformation of plasma ANP concentrations and the ventricular ejection fraction values in 62 cardiac patients (Fig. 4), suggesting that ANP concentrations may also be related to the degree of myocardial dysfunction.

ANP concentrations in aorta, pulmonary artery, and inferior vena cava. Plasma ANP was determined in 57 plasma samples collected from 10 patients during the hemodynamic study from both aorta and pulmonary artery. ANP values measured in the aorta (y ; 159.0 ± 163.7 ng/L) were very similar to those measured in the pulmonary artery (x ; 155.1 ± 154.3 ng/L) and showed a very close linear regression ($y = 1.66 + 1.04x$, $r = 0.977$, $P < 0.0001$). Moreover, from three of these patients (NYHA I) we also repeatedly measured the ANP concentrations in the inferior vena cava; the mean ANP value from the inferior vena cava (6.4 ± 6.2 ng/L) was greatly lower than the ANP measured in those patients' aorta (14.3 ± 16.7 ng/L) and pulmonary artery (22.6 ± 21.2 ng/L).

Discussion

The IRMA kit evaluated in this study showed good sensitivity and precision, comparable with those reported for other IRMA

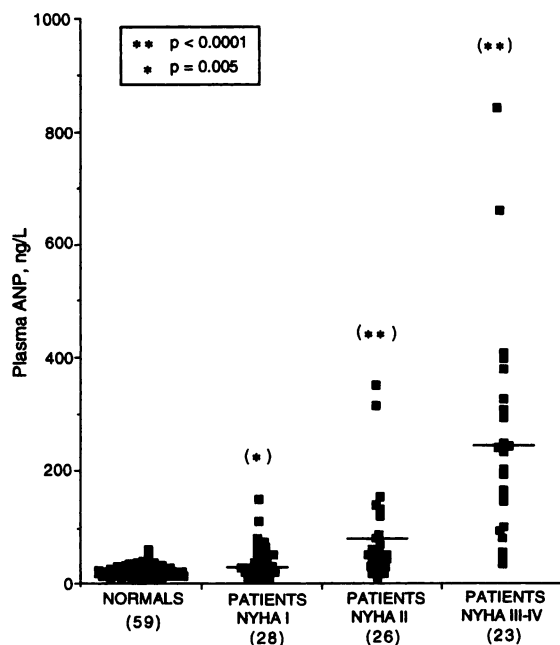


Fig. 3. ANP concentrations measured by the IRMA kit in control subjects and in three groups of cardiac patients, divided according to NYHA functional class.

The significance of differences calculated between the pairs of means of the four groups studied was tested by ANOVA ($P < 0.0001$) and Scheffé's test (with logarithmic transformation of data). The significance values shown are for differences from the healthy control group. The mean value of each group is indicated by a horizontal line, and the number of patients in each group is shown in parentheses.

methods [10, 11]. Furthermore, our results indicate that assay precision and sensitivity can be further increased by using a longer time (12 h overnight) and a lower temperature ($4-8$ °C) for the first incubation than suggested by the manufacturer. We could also increase the minimum amount of ANP measured per tube by assaying a greater volume of plasma than the $100 \mu\text{L}$ suggested by the manufacturer.

Using the IRMA kit, we were able to assay the circulating concentrations of ANP in all of the control subjects with acceptable precision. Available RIA methods frequently do not permit precise measurement of ANP concentrations in plasma samples from healthy controls [8, 9]. Indeed, as we previously reported [8, 9], some commercially available RIA kits, evaluated in our laboratory by the same procedures reported here, had detection limits between 10 and 20 ng/L, whereas that of the IRMA kit was ~ 2 ng/L (i.e., 5–10 times better than RIA). Moreover, the between-assay imprecision (CV, %) of RIA methods was generally $>20\%$ [8, 9], vs a between-assay imprecision of the IRMA kit of 6.7–11.4% for four different quality-control plasma pools. Finally, the RIAs generally required a preliminary extraction of plasma samples and more sample volume ($\sim 1-3$ mL) for the assay [8, 9] than did the IRMA kit ($50-300 \mu\text{L}$) (Fig. 2). Consequently, our data clearly demonstrate that the IRMA is preferable to RIAs for the routine assay of plasma ANP.

Our study shows that a well-standardized protocol should be used in the routine determination of plasma ANP by immunoassay, because sample collection and storage may greatly affect the plasma ANP concentrations. In particular, blood samples should be centrifuged immediately and the plasma should be separated and stored at -20 °C as soon as possible, because of findings that some blood cells (probably platelets, which share ANP receptors) [19, 20] can degrade ANP even in presence of plasma protease inhibitors. Plasma samples with evident hemolysis should be discarded and not assayed.

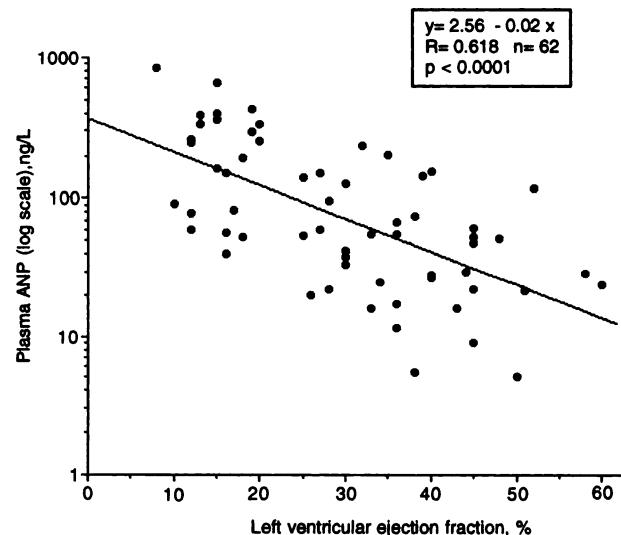


Fig. 4. Relation between logarithmically transformed concentrations of plasma ANP and ventricular ejection fraction (measured by equilibrium radionuclide angiography) in 62 patients.

The clinical results obtained with the IRMA kit agreed well with those by previously described RIA and IRMA methods [8–11, 21–27]. The reference range for healthy subjects is similar to that generally reported by the most accurate methods, e.g., other IRMAs [10, 11] or RIAs that use a preliminary extraction and purification of plasma samples with chromatographic procedures [2–4, 9, 21].

Our findings also confirm that the circulating concentrations of ANP were increased in patients with cardiac failure [5–7]. Indeed, plasma ANP increases with the progression of clinical severity of the disease, so some patients with only mild symptoms of diseases (NYHA I or II) and (or) initial myocardial involvement (ejection fraction >50%) could have ANP concentrations within the reference range. Cardiac patients with more severe symptoms of heart failure (NYHA III–IV) and myocardial dysfunction (ejection fraction <25%) currently show greatly increased values—as much as 30 times the mean value for healthy subjects (Figs. 3 and 4).

The main analytical characteristics of the IRMA (high sensitivity and precision, low assay volume) make it possible to accurately measure ANP concentrations during dynamic tests or pathophysiological studies, in which only a few milliliters of blood can be withdrawn, e.g., when several blood samples must be collected in a short period of time, or several analytes must be assayed in each sample. Indeed, we used the IRMA kit to simultaneously and repeatedly measure ANP concentrations in two (or more) different sites of circulation in the same patient. Our results are in complete agreement with several reports of data [28–33] showing nearly superimposable values of ANP plasma concentrations measured either in the pulmonary artery or in the aorta, whereas these ANP concentrations differed greatly from those in the inferior vena cava, confirming the important role of peripheral tissues in ANP clearance.

In conclusion, the IRMA kit we evaluated for ANP determination showed a good sensitivity, precision, and practicability and gave results that were superimposable on those obtained with previously described IRMAs [10, 11] but much better than those obtained by RIAs requiring a preliminary chromatographic extraction and purification of plasma samples [8, 9]. Moreover, the clinical results observed with this method were well in agreement with those found with other accurate methods (IRMAs or RIAs with preliminary chromatographic purification). Consequently, we prefer the IRMA over RIA for measuring plasma ANP, both for experimental studies and for routine assays in patients with heart failure.

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