

Measurement of Cardiac Natriuretic Hormones (Atrial Natriuretic Peptide, Brain Natriuretic Peptide, and Related Peptides) in Clinical Practice: The Need for a New Generation of Immunoassay Methods

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Background: Cardiac natriuretic hormones (CNHs) are a family of related peptides, including atrial natriuretic peptide (ANP), brain natriuretic peptide (BNP), and other peptides derived from the N-terminal portion of the proANP and proBNP peptide chains. Assays for cardiac natriuretic peptides have been proposed to help assess clinical conditions associated with expanded fluid volume. In particular, the assays can be useful for distinguishing healthy subjects from patients in different stages of heart failure. Measurements of these hormones have also been considered for prognostic indicators of long-term survival in patients with heart failure and/or after acute myocardial infarction. The different CNHs differ in their production/secretion patterns and have different clearance rates. Furthermore, there are numerous proposed assay configurations for each of these hormones, and it is not clear which assay provides the best pathophysiological and/or clinical information.

Approach: Here we review recent studies concerning the competitive (such as RIA, enzyme immunoassay, or luminescence immunoassay) and noncompetitive immunoassays (such as two-site IRMA, ELISA, or immunoluminometric assay) for the different cardiac natriuretic peptides to compare the analytical characteristics and clinical relevance of assays for the different CNHs and the different assay formats.

Content: Developing sensitive, precise, and accurate immunoassays for cardiac natriuretic peptides has been

difficult because of their low concentrations (on average, ~3–6 pmol/L) in healthy subjects and because of their structural, metabolic, and physiological characteristics. Competitive assays have historically suffered from lack of sensitivity and specificity for the biologically active peptides. These usually require tedious extraction procedures prior to analysis. Recently, immunometric assays have been developed that have improved sensitivity and specificity; it appears these will be the methods of choice.

Summary: To date, there is no consensus on the best assay procedure of cardiac natriuretic peptides. To facilitate widespread propagation of determination of these hormones in routine clinical practice, it will be necessary to study the new generation of noncompetitive immunometric methods that are less time-consuming and more sensitive and specific. Although several studies suggest that BNP exhibits better clinical utility than the other CNHs, more studies examining multiple CNHs in the same cohorts of patients will be necessary.

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The mammalian heart synthesizes and secretes a family of related peptide hormones (cardiac natriuretic hormones, CNHs),¹ which have potent diuretic, natriuretic, and vascular smooth muscle-relaxing effects as well as complex interactions with the hormonal and nervous systems (1–4). CNHs include atrial natriuretic peptide (ANP), brain natriuretic peptide (BNP), and their related peptides, whereas other natriuretic peptides, such as C-type natriuretic peptide (i.e., CNP) and urodilatin, structurally related to the ANP/BNP peptide family, are not produced

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¹ Nonstandard abbreviations: CNH, cardiac natriuretic hormone; ANP, atrial natriuretic peptide; and BNP, brain natriuretic peptide.

and secreted by cardiomyocytes but by other tissues (3, 4).

Several studies suggest the importance of measuring the circulating concentrations of CNHs in the classification and/or prediction of mortality/survival rates in patients with heart failure (1–4). In particular, a CNH assay might reduce the need for more expensive and invasive cardiac investigations in patients with cardiac disease (1–5). Because of the clinical relevance of CNH measurement, several different procedures have been proposed (3, 4). However, as yet the best procedure (gold standard) has not been ascertained, because CNH determination by competitive immunoassay methods is affected by several analytical problems (3, 4).

After we summarize the technical characteristics and potential clinical indications of various immunoassay methods for CNHs, their analytical performance and clinical relevance will be discussed and compared. Suggestions will be made for their use in clinical practice and for development of a new generation of CNH immunoassays.

MEASUREMENT OF ANP, BNP, AND RELATED PEPTIDES: WHICH PEPTIDE SHOULD BE MEASURED?

As reviewed recently (3, 4), developing a sensitive, precise, and accurate method for CNH assay has been difficult because of the structural, metabolic, and physiological characteristics of these hormones. Therefore, several important points should be taken into account when discussing the pathophysiological relevance of a particular CNH assay:

- The goal of a pathophysiological and/or clinical study should be to assess the activity of a specific hormone system; therefore, only the biologically active substances of this system should be assayed. As a consequence, only immunoassay methods using antibodies specific for the biologically active portion of the hormone (i.e., the part of the peptide chain recognized by the specific receptor) should be chosen to obtain a close relationship between biological and immunological activity (3, 4).
- Although ANP and BNP bind to the same specific receptors, they have different types of metabolism and spectra of biological activity, and their production and secretion may be regulated differently in humans (3, 4, 6, 7). It has been suggested that there may be different pools of intracellular natriuretic peptides that can respond separately to the same hemodynamic events (e.g., overload for ANP) or to the same pathology-related factors (e.g., cardiac hypertrophy for BNP) (7).
- ANP is stored in the atrial granules predominantly as proANP_{1–126}, which is split into a 98-amino acid N-terminal fragment (i.e., N-terminal proANP_{1–98}), and ANP in equimolar amounts (1–4). Because N-terminal proANP_{1–98} has a slower clearance than the biologically

Table 1. Mean detection limit, normal values (mean ± SD), and range (minimum and maximum values) of some competitive (EIA) and noncompetitive (IRMA and ELISA) immunoassays for CNHs.

Method ^a	Detection limit, pmol/L	Normal values, pmol/L	Range, pmol/L
IRMA ANP	0.73	5.6 ± 3.6	0.2–16.6
IRMA BNP	0.75	2.9 ± 2.7	0.1–12.4
ELISA proANP _{1–98}	76.9	731 ± 628	43–1502
IRMA proANP _{1–98}	40.5	228 ± 99	63–422
EIA proANP _{1–30}	9.5	708 ± 251	44–1289
EIA proANP _{31–67}	38.4	1422 ± 790	193–3339
EIA Nt-proBNP	13.6	246.8 ± 120.1	64–488
EIA Mid-proBNP	4.0	117.5 ± 100.3	0.2–368

^a ANP was measured by a previously described IRMA method (17, 19). BNP was measured by a previously described IRMA method (19, 20). The following methods were from Biomedica Gruppe (Vienna, Austria): ELISA proANP_{1–98} method (code BI-20892); EIA proANP_{1–30} method (code BI-20802); EIA proANP_{31–67} method (code BI-20822); EIA Nt-pro BNP method (code BI-20852), which uses an antiserum against the N-terminal proBNP_{8–29} peptide fragment; and EIA Mid-proBNP method (code BI-20862), which uses an antiserum against the N-terminal proBNP_{32–57} peptide fragment. IRMA proANP_{1–98} was kindly supplied by Shionogi and Co., Ltd. (Osaka Japan), described previously (24).

active peptide, its plasma concentration is higher (up to 10–50 times) than those of ANP and BNP (Table 1) (3, 4). Even if the N-terminal proANP_{1–98} is biologically inactive, several studies (8–11) have indicated that some peptides, produced in vivo by the degradation of this peptide, are biologically active. These peptides have been named the long-acting natriuretic peptide (i.e., N-terminal proANP_{1–30}); the vessel dilator peptide (i.e., proANP_{31–67}); and the kaliuretic peptide (i.e., proANP_{79–98}). However, these N-terminal proANP hormones exhibit (at least in part) different biological properties as well as different mechanisms of action (i.e., binding to different receptors) with respect to the ANP/BNP system (11, 12). Like ANP, BNP is also produced in the prohormone form (proBNP_{1–108}), which before the secretion by cardiomyocytes is split into the inactive N-terminal fragment proBNP_{1–76} and the biologically active hormone BNP (i.e., COOH-terminal proBNP_{77–108}) (3, 4, 12).

- CNHs are degraded both in vivo and in vitro by several proteases. EDTA and protease inhibitors (such as aprotinin) are generally added to whole blood samples to inhibit this degradation; plasma samples are then frozen and stored at –20 °C as soon as possible (3, 4). This expensive and time-consuming procedure renders the use of the CNH assay in clinical routine practice difficult. However, recent studies have suggested that the use of protease inhibitors may be not necessary, at least for BNP and proANP_{1–98} (13). If these findings are confirmed, the BNP and proANP_{1–98} assays may be preferable to the ANP assay for routine clinical practice.

MEASUREMENT OF CNHS AND RELATED PEPTIDES BY IMMUNOASSAY METHODS

The determination of CNHs and related peptides is generally performed by means of competitive immunoassays, such as RIA or EIA; recently some noncompetitive immunoassays have been developed (3, 4). The analytical characteristics of immunoassays for ANP and BNP and those for N-terminal fragments of proANP and proBNP will be discussed separately in the following two sections because of their different analytical characteristics.

ANP AND BNP METHODS

There is no agreement on the best procedure for measuring ANP and BNP, because determination by competitive immunoassay methods is affected by several analytical problems. In particular, specific (i.e., metabolites or other peptides structurally related to CNHs) or nonspecific (such as plasma or cellular proteins binding CNHs) interferences may affect the assay (3, 4). Moreover, an assay detection limit of <1 pmol/L is required for the measurement of ANP and BNP concentrations in healthy subjects with acceptable precision (Table 1) (3, 4). For these reasons, preliminary extraction and/or chromatographic purification of large volumes (>1 mL) of plasma (or tissue extract) is required for eliminating these interferences, thus increasing accuracy (i.e., specificity) and sensitivity of the determination of competitive immunoassay methods (such as RIA or EIA) (4).

Noncompetitive immunometric assays for the measurement of ANP and BNP have been developed recently to overcome the problems encountered with competitive assays (3, 4). The methods of this second generation are generally "two-site" (sandwich) immunometric assays, using two specific monoclonal antibodies or antisera prepared against two sterically remote epitopes of the ANP (14–17) or BNP (18–20) peptide chain.

As reviewed recently (3, 4), noncompetitive immunoassay methods have several advantages over competitive assays. Noncompetitive assays are generally 5–20 times more precise and sensitive than their respective competitive assays and are not significantly affected by nonspecific or specific interference. Therefore, noncompetitive immunoassays methods for ANP and BNP do not usually require preliminary extraction and purification of the sample and also use a lower plasma volume (generally, 0.05–0.3 mL) than competitive assays (4). Furthermore, noncompetitive immunometric assays generally have a larger working range than that of competitive assays (4). These facts suggest that noncompetitive immunoassays for ANP and BNP may be more suitable for clinical routine than competitive assays (4).

ASSAYS FOR THE N-TERMINAL proANP AND proBNP

Theoretically, developing an immunoassay for N-terminal peptide fragments of proANP and proBNP should be easier than that for ANP and BNP, because these peptides have higher plasma concentrations (Table 1). However,

immunoassays for N-terminal peptide fragments of proANP and proBNP may also be affected by several analytical problems, mainly concerning the assay specificity.

The intact N-terminal proANP_{1–98} is a long peptide; this implies that each anti-N-terminal proANP antibody recognizes only one epitope of the peptide. Consequently, because low-molecular mass fragments of N-terminal proANP_{1–98} are present within the circulation, different competitive immunoassays, using different antibodies, may give different results (3, 4), as seen in Table 1.

EIA methods that use antisera specific for different epitopes of N-terminal proANP_{1–98} peptide chain usually show cross-reactions close to 100% with the intact proANP_{1–98} peptide (3, 4). Even if the values found with these EIAs are highly correlated when compared ($r = 0.917$; $n = 115$; $P < 0.0001$), however, these methods show significantly different clinical results and reference values (Table 1).

These data agree closely with the results of other studies (21–23), indicating that RIAs for the assay, respectively, of the long-acting natriuretic peptide, the vessel dilator peptide, and the kaliuretic peptide actually also recognize the whole N-terminal proANP_{1–98}.

It is theoretically conceivable that a two-site noncompetitive immunometric assay, using two different monoclonal antibodies specific for two sterically remote epitopes of intact N-terminal proANP_{1–98}, would be more suitable than a competitive method to measure the intact N-terminal proANP_{1–98}, with only minor interference from its degradative fragments, as demonstrated recently for an IRMA method (24).

In conclusion, competitive and noncompetitive immunoassay methods for the N-terminal proANP_{1–98} may measure, at least in part, different substances, which probably have different biological activities and metabolic pathways and thus different clinical relevance (8–11).

Noncompetitive assays for the determination of the N-terminal proBNP peptide, which use antisera or antibodies specific for different epitopes of the peptide chain, could also have different results, as demonstrated by the comparison of two EIA methods (Table 1). Recently, two noncompetitive immunoluminometric assays for N-terminal BNP_{1–76} have also been described (25, 26). These immunoluminometric assay methods are highly sensitive (2–3 pmol/L) (25, 26) and specific for the intact peptide chain N-terminal BNP_{1–76} (25); furthermore, they could be easily modified for use in a fully automated system (25).

COMPARISON OF CLINICAL RELEVANCE OF IMMUNOASSAY METHODS FOR CNHS

Indications and usefulness of CNHs assays have been studied extensively for use in cardiovascular disease, especially in patients with various degrees of heart failure (1–4, 27–36). However, it must be emphasized that because CNHs are raised in a variety of clinical conditions (3, 4), a normal value has only negative predictive value,

whereas increased values usually call for further diagnostic investigation in patients with cardiovascular diseases (31, 32).

Studies comparing the clinical usefulness of different CNH assays in patients with different degrees of heart failure have produced conflicting results. In some studies, the assay for N-terminal proANP₁₋₉₈ peptides was shown to be equally or even more clinically useful than other CNH assays (27-29), whereas in others (30, 31, 33-36) BNP was found to be the best marker of myocardial involvement.

Although these conflicting results could be partly explained by a heterogeneous nature of groups studied, the different specificities of methods used to measure the CNHs could also play an important role. Unfortunately, a comparison of analytical and clinical performances of these assays is difficult because the analytical characteristics of methods used are not always specified in clinical studies.

The ANP assay could be more useful than that of other CNHs in some pathophysiological conditions. ANP is produced predominantly in atrial cardiomyocytes and can be promptly released from these cells after an acute stimulation (atrial stretch and overload) (7). Furthermore, ANP has a shorter plasma half-life (~3-5 min) than BNP

and especially intact N-terminal proANP₁₋₉₈ (3, 4). For these reasons, ANP should be a good marker of acute overload and/or rapid cardiovascular hemodynamic changes. Indeed, circulating concentrations of ANP decrease more after hemodialysis than those of BNP in patients with chronic renal failure (3, 4), whereas changes in plasma concentrations of intact N-terminal proANP₁₋₉₈ are less pronounced (Fig. 1). However, other causes should be also taken into account to explain the greater variations of ANP and BNP during hemodialysis compared with intact N-terminal proANP₁₋₉₈, such as the lower molecular weight of ANP and BNP than that of intact N-terminal proANP₁₋₉₈, allowing a better crossing through the hemodialysis membrane filter. Furthermore, ANP increases more than N-terminal proANP₁₋₉₈ during rapid ventricular pacing (37).

The assays of long-acting natriuretic peptide, vessel dilator peptide, and kaliuretic peptide, released simultaneously with ANP, should be also considered to be a marker of atrial stretch and overload.

Less information is available on the clinical relevance of the measurement of N-terminal BNP₁₋₇₆ compared with other CNH-related peptides (12, 25, 26); however, at present, this assay may have the same clinical indications as the intact N-terminal proANP₁₋₉₈ assay.

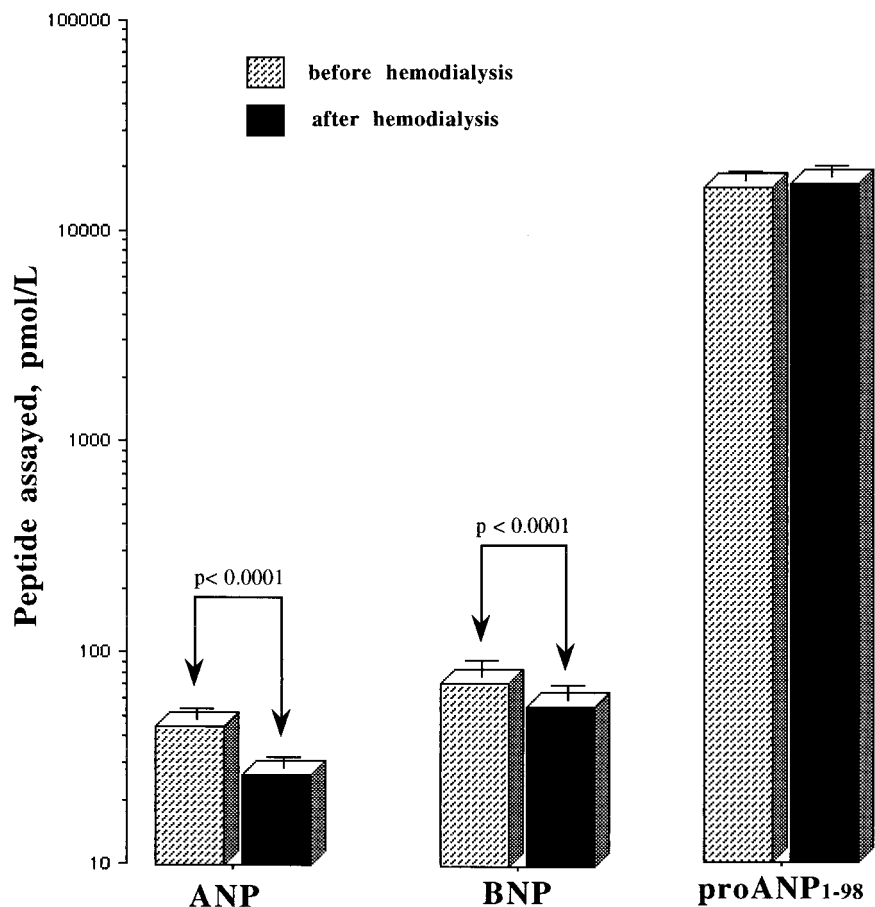


Fig. 1. Mean values (\pm SE) of ANP, BNP, and N-terminal proANP₁₋₉₈, measured in 51 patients with chronic renal failure by means of noncompetitive immunoassays before and after the hemodialysis session.

THE NEED FOR A NEW GENERATION OF IMMUNOASSAY METHODS

In Western countries, congestive heart failure is the most frequent hospital discharge for patients >65 years of age; in the United States alone, ~2.5 million patients suffer from heart failure (38). Several recent studies have underlined the clinical importance to routinely assay CNHs for classification, follow-up, and/or in prediction of mortality/survival rates of all patients with heart failure (1–5, 27–36).

This implies that reliable assays for CNHs must be available for all clinical laboratories; unfortunately, we are far from this goal. As reviewed previously in detail (4), developing a reliable assay for CNHs presents nearly insurmountable analytical problems. CNHs are a complex family of related peptides with both similar peptide chains and degradation pathways. Moreover, CNHs have greatly differing plasma concentrations (Table 1) and half-lives and may also undergo minor modifications in plasma, such as oxidation. For these reasons, several peptides may simultaneously interfere in an immunoassay, giving falsely high values. On the other hand, an assay that is very specific for a particular peptide may lead to underestimation, because peptide alteration may occur in the circulation, during specimen collection, or during storage.

Indeed, several methods for CNH assays have been described, but all have some problems concerning lack of sensitivity, precision, and/or accuracy (specificity). Furthermore, these methods, even when measuring similar or identical peptides, show different clinical results and reference values (Table 1) so that each laboratory has to determine its own reference interval.

CNHs and related peptides are generally measured with competitive immunoassay methods that use radioactive labels (i.e., RIA). The main advantage of RIAs compared with other immunoassays is the lower cost (4). The substitution of a RIA with a commercial noncompetitive immunoassay method may increase the cost for materials from two- to threefold (or more) in a laboratory, which has the opportunity to completely (or partially) set up an RIA without using expensive commercial products (i.e., the possibility of preparing specific antibodies and radioactive tracers directly) (4). However, radioactive tracers are less stable and safe than nonradioactive labels and can only be used in a few clinical laboratories.

Noncompetitive immunometric assays (such as some IRMAs) for CNHs generally have a better degree of sensitivity, precision, and specificity than the respective competitive immunoassays (such as RIA or EIA) (3, 4). Therefore, this second generation of immunoassay methods should be preferred in all laboratories interested in pathophysiological and clinical research on CNHs, requiring a maximum sensitivity, precision, and accuracy. However, these methods are still time-consuming (an assay typically requires from 12 to 36 h) and cannot be used in a fully automated analytical system.

Because at present there is no a general consensus on the best method for CNH assay, at the present time each

laboratory must choose the methods and the peptides (hormones) to assay that meet its own clinical requirements as well as to other issues, such as stability (of both analytes and materials), ease of measurement, and costs.

In our opinion, to allow a more widespread propagation of CNH assay in all clinical laboratories and routine clinical practice it is necessary to set up a new generation of noncompetitive immunometric assays that are more sensitive, precise, and easy to perform, do not use radioactive labels, and can be directly used in fully automated analytical systems. Indeed, some recently developed immunoluminometric assay methods for N-terminal proBNP_{1–76} show some of above-cited analytical characteristics (including their possible use in a fully automated system); therefore, they should be taken as a starting point for the development of a new generation of CNH immunoassays (25, 26). More recently, a rapid, fully automated method for BNP assay, which could be used for point-of-care testing of patients with congestive heart failure, has also been described (39).

Three main problems must be resolved to allow widespread propagation of CNH assay in clinical practice, including their use in emergency and primary care, as suggested by several authors (1–4, 31):

(a) An increase in analytical sensitivity should be achieved. This improvement should allow the determination of all the normal range of CNHs with an acceptable CV (<15%); this goal is particularly pressing for the BNP assay (4, 19, 20). As demonstrated recently for other hormone immunoassays (for example, for thyroid-stimulating hormone assay), an increase in the analytical sensitivity also produces an increase in functional (clinical) sensitivity (40).

(b) An increase in analytical specificity (accuracy) is also necessary, in particular for the assay of proANP_{1–98}- and proBNP_{1–76}-related peptides and hormones.

(c) An increase in practicability is also necessary. We think that it will not be possible to spread the routine measurement of CNHs in clinical practice, including in emergency and primary care, without a new generation of immunoassay methods that permit the determination of CNHs in a few hours (or even minutes).

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