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A Two-site Delfia Immunoassay for Measurements of the N-terminal Peptide of pro-Atrial Natriuretic Peptide (nANP)

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ABSTRACT

A rapid, sensitive and reliable two-site immunoassay for measurements of the N-terminal peptide of pro-atrial natriuretic peptide (nANP) is presented. The method uses one monoclonal antibody, directed against the N-terminal part of nANP, as catcher antibody and another monoclonal antibody, directed against the C-terminal part of nANP, as detector antibody. The catcher antibody is biotinylated and is bound to streptavidin pre-coated microtiter strips. The detector antibody is labelled with Europium, which is measured in a Wallac DELFIA time-resolved fluorometer. Blood collected in plain Vacutainer® tubes gave same measured amounts of nANP as blood collected in heparinised tubes. Blood collected in tubes containing EDTA gave same measured amounts of nANP as the plain tubes, provided that a 2-step assay protocol was used. Based upon 100 healthy blood donors, a reference interval was calculated to <450 pmol/L. Within the reference group there was a significant increase of serum nANP with age. Based on 42 patients with different degree of impaired renal function, a significant correlation of nANP and serum creatinine was found. Assay performance, given as total assay variation was 12%, 10% and 9% respectively at serum levels of 140, 970 and 3500 pmol/L. It is concluded that this method is fast, sensitive and reliable for clinical measurements of nANP.

INTRODUCTION

Atrial natriuretic peptide (ANP) is a 28-amino acid peptide hormone synthesised and stored in atrial myocytes as a prohormone. It is released upon various stimuli, the most important being increased atrial pressure and stretch of the myocyte fibres (2,7,11,14). Stimulated release and increased plasma levels of ANP is associated with chronic congestive heart failure and the circulating levels are proportional to the severity of the disease (4,12,13,15).

On secretion, the 126-amino acid prohormone is split and the active ANP hormone is released in equimolar amounts with a N-terminal peptide (nANP). Thus, measurements of nANP can be used to monitor ANP release (7,13,14). Measurements of nANP has several advantages compared to ANP measurements since the nANP-molecule has a longer half-life in the circulation and circulates at higher levels. nANP also is also more stabile in vitro and can be stored in plasma samples up to three days at room temperature without affecting the levels, compared to ANP which is rapidly degraded in vitro (8). Furthermore, some studies have reported that measurements of nANP are more reliable markers for cardiac failure than measurements of ANP (3,5,7,9). Thus, the aim of this study was to develop a fast and sensitive method for measurement of nANP suitable for clinical use and research.

MATERIALS AND METHODS

Chemicals:

Standard chemicals of pro analysi grade was used for preparation of buffers and reagents (Kebo Lab, Stockholm, Sweden).

Buffers:

Coating buffer: 0.05M Tris-HCl-buffer, pH 9.2, containing 0.15M sodium chloride and 0.02% sodium azide. Blocking buffer: 0.05M Tris-HCl-buffer, pH 7.8, containing 2% bovine serum albumin (BSA), 0.15M sodium chloride and 0.02% sodium azide. Biotinylation buffer: 50 mM sodium carbonate buffer, pH 9.6 containing 50-fold excess of biotin-isothiocyanate dissolved in dimethylformamide. Washing buffer: Wash concentrate containing Tris-HCl-buffer at pH 7.8, Tween 20 and Germall II (Wallac OY, Turku, Finland), diluted 25 times. Assay buffer: a standard Delfia assay buffer (DELFIA hTSH-ultra buffer, Wallac) was used for dilutions of antibodies and standards. Enhancement solution (Wallac): Used before fluorescens readings to liberate Europium (Eu) from the chelating complexes.

Antibodies:

One monoclonal antibody (Medix Biochemica OY, Kauniainen, Finland), directed against amino acids 1-30 in proANP, was used as catcher antibody and another monoclonal antibody (Medix Biochemica), directed against amino acids 79-98, was used as detector antibody. The catcher antibody was either coated directly to 96-wells microtiter plates or biotinylated (10) and bound to streptavidin pre-coated microtiter strips. The detector antibody was labelled with Eu according to purchaser's instructions (DELFIA Eu-labelling kit, Wallac).

Standard preparations:

Dilutions of pooled patient serum was used as standard. Initial standardisation was performed against a calibrated preparation of pooled serum kindly provided by Medix Biochemica. Dilutions of serum standards were performed in a serum pool pre-treated with activate charcoal in order to absorb cross-reacting peptides. A synthetic nANP peptide standard preparation covering the amino acids 1-30 coupled to amino acids 79-98 was used for comparison (Medix Biochemica).

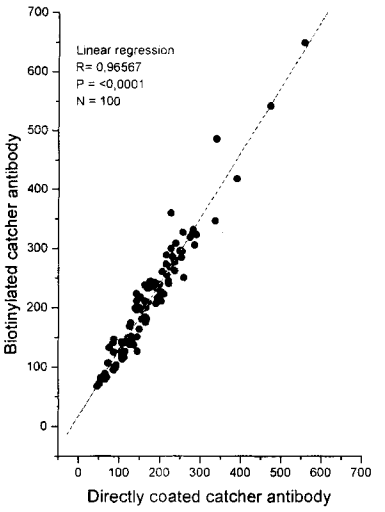

Fig. 1: Measurements of nANP in a reference population of 100 healthy blood donors. Assays performed with the catcher antibody directly coated to microtiter wells measured consistently 20% lower levels of nANP compared to assays where the biotinylated catcher antibody was use in combination with streptavidin pre-coated microtiter strips. However, the correlation between the methods was excellent (R=0,97, P<0,0001).

Coating of microtiter plates

The catcher antibody was dissolved in coating buffer to a concentration of 1 µg/mL and 200 µL was added to each well. Incubation was performed at +4°C for 24 h. The wells were then emptied, incubated with 300 µL of blocking buffer at +4°C for 24 h and finally washed with washing buffer. The microtiter plates were stored at +4°C at humid conditions until use.

Biotinylation

The catcher antibody (2 mg) was dissolved in biotinylation buffer and incubated for 3 hours at room temperature (10). The biotinylated antibody was separated from excess reagents by 2 gel

filtration separations, first on a NAP-5 and then on a NAP-10 column (Pharmacia, Uppsala, Sweden) using the separation buffer. Using 50-fold molar excess of biotin-isothiocyanate, the degree of biotinylation was 97%. The final biotinylated antibody preparation was stored with 0.1% BSA as a stabilising agent.

Europium labelling

The detector antibody was labelled with Eu according to purchaser's instructions (DELFIA Eu-labelling kit, Wallac). The labelling degree was 15 moles of Eu/IgG molecule. The final Eu-labelled antibody preparation was stored with 0.1% BSA as a stabilising agent.

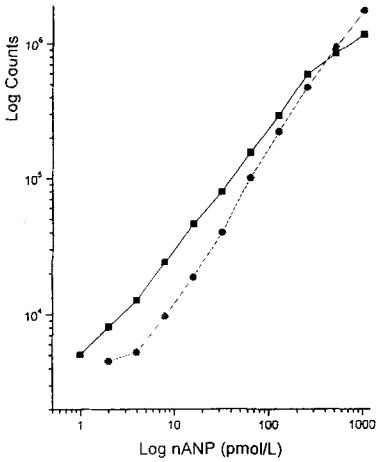

Fig. 2: Standard curves constructed from a synthetic peptide standard (■) and from pooled patient serum (●). Dilution curves are not parallel.

The nANP assay:

A Delfia sandwich immunoassay for measurements of nANP was developed and effects of different assay modifications were investigated:

1. Direct coating of the catcher antibody to microtiter wells was compared with biotinylation of the catcher antibody, used in combination with microtiter strips pre-coated with streptavidin (Wallac).
2. Standard preparations of pooled patient serum was compared to a synthetic standard peptide. Also the effects of diluting standard preparations in assay buffer or charcoal-treated serum was investigated.
3. Optimal incubation times and comparison of one-step versus two-step incubation protocols.

Blood samples

Serum samples from 100 healthy blood donors, 63 men and 37 women at ages from 19 to 69 years, were collected for establishment of a reference interval. Serum samples from 42 patients with different degrees of renal dysfunction were collected for investigation of the relations of serum creatinine and nANP-levels. Blood from 20 patients was collected in plain Vacutainer® tubes or tubes containing either Heparin or EDTA for investigation of effects of different additions during blood sampling.

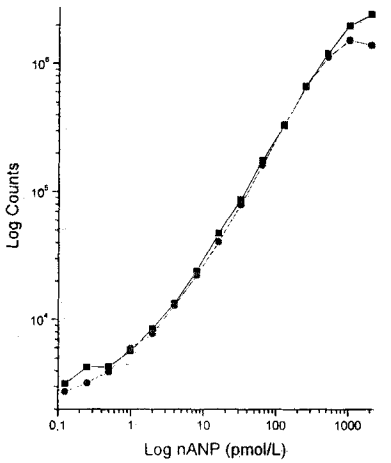

Fig. 3: Standard curves from a two-hour one-step incubation (●) and a two-step two + two-hour incubation (■) are parallel within the working area.

RESULTS

Assay performance:

Assays performed with the catcher antibody directly coated to microtiter wells measured consistently 20% lower levels of nANP compared to assays where the biotinylated catcher antibody was use in combination with streptavidin pre-coated microtiter strips (Fig. 1). However, the correlation between the methods was excellent (R=0,97, P<0,0001). Dilutions of the synthetic peptide standards and the serum pool standards were not parallel (Fig. 2). Dilutions of the both standard preparations in buffer were not parallel to dilutions in charcoal-treated serum (not shown), indicating presence of matrix effects. The charcoal-treated serum did not contain any detectable amounts of nANP. The Eu-counts at a given concentration were not increased further after 1.5 h of incubation. Incubation of standards or unknown samples with the catcher antibody for 2 h, followed by 6 times washing and a 2 h incubation with the detector

antibody (two step protocol) gave similar results compared to simultaneous incubation with all reagents for 2 h (one step protocol) (Fig. 3).

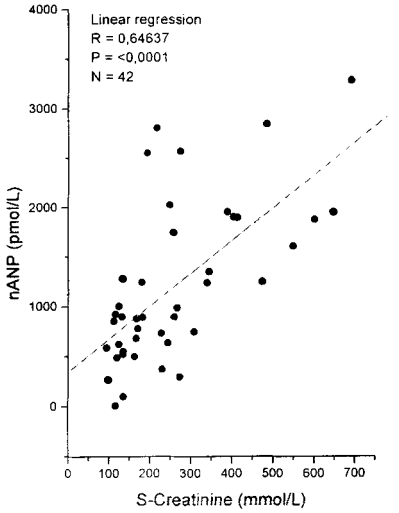

Fig. 4: Relationship between serum creatinine and nANP. There is a significant increase of nANP levels with increasing creatinine levels (R=0,64, P<0,0001, N=42).

Measurements of blood samples:

Blood collected in plain Vacutainer® tubes gave identical results as the ones collected in heparinased tubes, using both one-step and two-step protocols. Blood collected in EDTA tubes could not be measured in the one-step assay since EDTA bind Eu and thus interfere with the fluorescent detection. However, when the two-step assay was used, the EDTA plasma levels were comparable to the serum levels of nANP. There was a significant correlation between serum creatinine and nANP levels (Fig. 4). The reference range for all 100 blood donors was calculated to <450 pmol/L (mean ± 2 SD). However, there was also a significant increase of nANP with age and in the subgroup of blood donors older than 40 years the reference range was <550 pmol/L, compared to <350 pmol/L for the blood donors younger than 40 years of age (Fig. 5).

Final set-up of method

Considering the results presented above, the final set-up of the method was as follows; A calibrated serum pool was used for standard preparations. Dilutions of serum was performed in charcoal treated serum. Twenty µL of standards or unknown samples were first added to microtiter wells precoated with streptavidin, followed by 80 µL assay buffer. Then 100 ng of

biotinylated catcher antibody and 200 ng of Eu-labelled detector antibody (50 µL of each, diluted in assay buffer) were added to each well. The incubation was performed in room temperature under continuous shaking during 2 h. After 6 times washing, 200 µL enhancement solution was added, followed by 15 min incubation. Final reading of results was performed on a DELFIA fluorometer (Wallac) and the results were calculated on a MultiCalc program (Wallac), using spline function to construct the standard curve. For measurements of blood samples taken in tubes containing EDTA, a 2 + 2 h incubation time protocol was used.

Assay performance, given as within assay variation, was 8%, 4% and 6% at serum levels of 140, 970 and 3500 pmol/L respectively (n=32). The total assay variation was 12%, 10% and 9% respectively at the these serum concentrations (n=32).

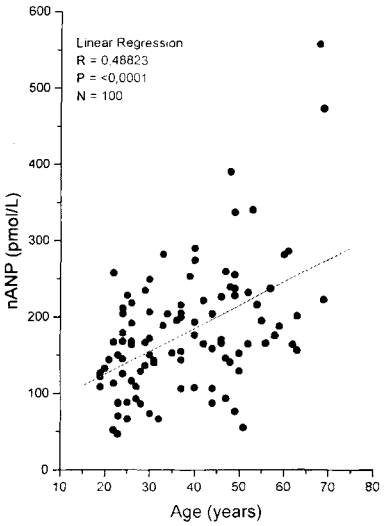

Fig. 5: Relationship between age and nANP. There is a significant increase of nANP levels with increasing age (R=0,49, P<0,0001, N=100).

DISCUSSION

This is the first two-site Delfia immunoassay for measurements of the N-terminal peptide of pro-Atrial Natriuretic Peptide (nANP). Optimised as outlined above, the method is capable of fast and reliable determinations of serum nANP levels.

Synthetic standard preparations would have been be ideal for standardisation of the method. However, we found that dilution curves of the synthetic standard were not parallel to dilutions of serum samples with high levels of nANP. The synthetic standard was only 50 amino acid long compared to the native 98 amino acid long nANP-molecule. We therefore anticipate that the different binding ability of the antibodies can result from more pronounced steric hindrance when two 150 kDa antibodies bind simultaneously to the about 6 kDa synthetic peptide compared to binding to the about 12 kDa native nANP molecule. This could be a significant problem when adapting two-site sandwich techniques for measurements of small peptides to replace the competitive radioimmunoassay techniques.

Dilutions of standard preparations and high serum samples in assay buffer were not parallel to dilutions in serum. This implies influence of serum components to antibody binding and shows the necessity of minimising matrix effects by using a similar matrix in standard preparations and in unknown samples. However, it is often difficult to find a "true" blank with similar matrix. Charcoal treatment is a well known method to absorb peptides from serum and this technique was successfully used to eliminate nANP from serum samples to provide the serum blank.

Assays performed with the catcher antibody directly coated to microtiter wells measured consistently lower levels of nANP compared to assays where the biotinylated catcher antibody was use in combination with streptavidin pre-coated microtiter strips. These results are most likely due to the fact that the directly coated antibody may change its 3-dimensional structure upon coating, which can affect the antigen-binding reaction negatively. Furthermore, with the biotinylated antibody technique the binding reactions are performed in liquid phase, rather than in liquid-solid phase as in the case with directly coated antibody, which also facilitates the binding reactions.

The reference range of this method is comparable to other methods for measurements of nANP and proANP (6) Also the influence of impaired kidney function and increase with age has been reported before (1).

It is concluded that this method is fast, sensitive and reliable for measurements of nANP and thus suitable for research and further clinical studies.

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