An immunoluminometric assay for N-terminal pro-brain natriuretic peptide: development of a test for left ventricular dysfunction

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ABSTRACT

Measurement of plasma levels of brain natriuretic peptide (BNP) has been used to assess left ventricular dysfunction and prognosis. Levels of the N-terminus of the precursor of BNP (NT-proBNP) have been reported to be elevated to a greater extent than BNP in left ventricular dysfunction. We have devised a non-radioactive sensitive and specific assay for NT-proBNP based on a competitive ligand binding principle. The chemiluminescent label 4-(2-succinimidyl-oxycarbonylethyl)phenyl-10-methylacridinium 9-carboxylate fluorosulphonate was used to label peptides representing domains in the middle and C-terminal sections of NT-proBNP. Assay of the C-terminal section of NT-proBNP (amino acids 65–76) in patients with proven left ventricular dysfunction [left ventricular wall motion index median 0.9 (range 0.3–1.4)] revealed elevated values [median 639 (386–911) fmol/ml] compared with normal controls [left ventricular wall motion index of 2 in all, NT-proBNP median 159 (120–245) fmol/ml, \( P < 0.001 \)]. Measurement of the middle section of NT-proBNP (amino acids 37–49) was not a discriminating test. It is thus possible to derivatize small peptides with a methyl acridinium label and preserve immunodetection with specific antibodies. Such methodology may allow non-radioactive immunoluminometric assays to be devised.

INTRODUCTION

Brain natriuretic peptide (BNP) is a 32-amino-acid peptide [1] that is synthesized predominantly in the left ventricle as the 108-amino-acid prohormone proproBNP (\( \gamma \)-BNP) [2–4]. The hormone is a potent vasodilator and natriuretic factor regulating salt and water homeostasis. Recently, interest has focused on the use of hormonal markers such as BNP, atrial natriuretic peptide (ANP) and N-terminal proANP (NT-ANP) as indices of left ventricular dysfunction (LVD) and prognostic indicators of outcome after acute myocardial infarction (AMI) [5]. In LVD and after AMI, BNP synthesis is increased in both the infarcted and non-infarcted myocardium [6]. Thus the secretion of this peptide may reflect not just LVD but may be a more sensitive index of abnormal wall stress preceding the process of ventricular remodelling [7,8]. Recent studies have indicated that elevated levels of ANP, NT-ANP (the N-terminal fragment of the ANP prohormone) and BNP are powerful predictors of cardiovascular mortality after AMI [5,9]. Of these measures, BNP may have the greatest potential to complement the standard prognostic indicators for risk stratification in patients with LVD [10]. The development of these plasma assays for hormones that may be elevated in LVD may lead to non-invasive techniques for assessing ventricular function which may be useful both for the diagnosis of LVD and monitoring of the response to medical therapy.

Circulating concentrations of BNP are raised in LVD

Key words: assay, brain natriuretic peptide, chemiluminescence, heart failure, plasma.

Abbreviations: AMI, acute myocardial infarction; ANP, atrial natriuretic peptide; BNP, brain natriuretic peptide; CNP, C-type natriuretic peptide; ILMA, immunoluminometric assay; LVD, left ventricular dysfunction; LVWMI, left ventricular wall motion index; NT, N-terminal; RLU, relative light units; TFA, trifluoroacetic acid; WMI, wall motion index.

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after myocardial infarction and in chronic heart failure [8,11,12]. It has been suggested that circulating BNP may be used as a marker of asymptomatic LVD in patients with suspected or known cardiac disease [13]. Two studies have shown that BNP is a good indicator of left ventricular systolic dysfunction in the general population. Cowie et al. [14] demonstrated that in patients presenting to a general practitioner with suspected heart failure, plasma BNP can reliably identify those who merit further investigation. A larger study by McDonagh et al. [15] also showed that plasma BNP could be used to screen the general population for left ventricular systolic dysfunction, especially when the test is targeted at high-risk groups.

However, the standard assays for plasma BNP measure both BNP and its precursors with no separation of the components into the high-molecular-mass proBNP and lower molecular mass active BNP-32 forms, even though more detailed studies using gel permeation chromatography of these plasma extracts have clearly demonstrated the presence of these two components [2–4]. Furthermore, recent work has reported the existence of NT-proBNP (an 8.6 kDa peptide, the N-terminal 76 amino acids of proBNP). NT-proBNP has been shown to circulate in plasma at higher concentrations than BNP-32 in patients with cardiac failure [16] and is elevated even in NYHA class I congestive heart failure [17]. A recent study suggested that the measurement of NT-proBNP is an equally sensitive indicator of LVD after AMI when compared with BNP-32 [18].

All of the above assays for BNP, ANP or their precursors used radiolabelled ligand in a competitive binding assay format. Such ligands are often unstable and have to be utilized before radioactive decay renders them unusable. Moreover, radiation protection and disposal need to be considered and add to the cost. We describe below the development of a competitive immunoluminometric assay (ILMA) for NT-proBNP since this ligand has been described as circulating in high concentrations in LVD [16–18]. Although the labelling of antibodies with the chemiluminescent label 4-(2-succinimidyloxycarbonyl ethyl)-phenyl-10-methylacridinium 9-carboxylate fluorosulphonate was a gift from Drs Stuart Woodhead and Ian Weeks, Molecular Light Technology Ltd, Cardiff, U.K. The paramagnetic particles coated with goat anti-rabbit IgG were from Metachem Diagnostics Ltd, Northampton, U.K. The C$_{18}$ plasma extraction columns and the peptides ANP, BNP, C-type natriuretic peptide (CNP) and proBNP (22–46) were obtained from Peninsula Laboratories, Merseyside, U.K. The nitrocellulose (0.2 μm) for peptide blotting using tricine gels was from Schleicher and Schuell, Dassel, Germany. The peptide molecular mass markers, tricine and all other reagents of Analar grade were obtained from Sigma Chemical Co. Ltd, Poole, Dorset, U.K.

**Production of antibodies**

The NT1 and NT2 peptides were conjugated to haemocyanin with the hetero-bifunctional cross-linker ε-maleimidocaproyl acid N-hydroxysuccinimide ester. Haemocyanin was dissolved in buffer (10 mg/ml in 100 mmol/l Na$_2$HPO$_4$ buffer, pH 8) and 1 ml was rapidly mixed with 400 nmol of ε-maleimidocaproyl acid N-hydroxysuccinimide ester (in 10 μl of dimethylformamide). After 30 min, the derivatized haemocyanin was gel-filtered on a Sephadex G25 column conditioned with 100 mmol/l Na$_2$HPO$_4$ buffer, pH 7.4. The haemocyanin was then mixed with 1 mg of NT1 or NT2 and the conjugation reaction proceeded for 3 h at room temperature. The conjugates were then dialysed with PBS for 3 days at 4 °C, with four changes of the PBS.

Two rabbits were injected subcutaneously with antigens (1 mg) emulsified with complete Freund’s adjuvant. After a month, booster injections (1 mg) were given intravenously every 2 weeks and the antisera obtained after 3 months. The IgG fraction was obtained by Protein A-Sepharose chromatography. Antibody G172 reacted with the peptide NT1 and G185 with NT2.

**Peptide labelling with the methyl acridinium ester**

The peptides NT1 and NT2 were dissolved in 100 mmol/l Na$_2$HPO$_4$ buffer, pH 8, at a concentration
of 200 μmol/l and 100 μl was pipetted into an Eppendorf tube. Five micrograms of the methyl acridinium ester was dissolved in 5 μl of dimethylformamide and mixed with 20 nmol of the peptide to be labelled. After incubation at room temperature for 30 min in the dark, 100 μl of a lysine quench solution (10 mg/ml in 100 mmol/l Na₃HPO₄ buffer, pH 8) was added and incubated for another 5 min. The labelled peptide solution was acidified with an equal volume of 1% trifluoroacetic acid (TFA). An aliquot of this was then injected on to a 3.9 mm × 150 mm Deltapak C₁₈ 300 Å column, mounted within a HPLC system (Waters, Watford, Herts, U.K.) consisting of a Waters 600S controller, 626 pump and 486 tuneable absorbance detector set at 215 nm to detect ultraviolet absorbance of the peptide peaks. The column was equilibrated with 0.1% TFA and a gradient of acetonitrile and the recovery of these labelled tracers amounted to 27.5 ± 2.8% of the total label used. The hydrophobicity of the labelled peptides after derivatization with the methyl acridinium ester facilitated the separation from unlabelled peptide. These fractions were collected and used for development of the ILMAs. Fractions collected at other times (corresponding to 215 nm absorbance peaks) were inactive. The labelled peptides were stored in the dark at −70 °C in the 0.1% TFA buffer, being stable for over 6 months. One preparation produced enough tracer for about 10 000 experiments.

Subjects
Blood was taken from 12 normal control subjects and 12 patients with LVD after informed consent was obtained. The studies were approved by the Leicester Health Authority Ethics Committee. Normal controls were outpatients who were scanned in the echocardiography department for suspected murmurs but were found to have no significant cardiovascular abnormality. Wall motion index (WMI) was measured using a nine-segment echocardiographic model (see below) and used to assess left ventricular function. All 12 normal controls [five males, median age 38 (20–79) years] had a WMI of 2. None was on any treatment. LVD was defined as a WMI ≤ 1.4 and the 12 patients with LVD [five males, median age 72 (56–87) years] had median WMI measurements of 0.9 (0.3–1.4). Two patients with LVD were categorized within NYHA class I, three patients within class II, four patients within class III and three patients within class IV. Five of the patients had ischaemic heart disease and seven had hypertension. Mitral regurgitation as assessed by echocardiography was present in 11 patients. Eight patients were on loop diuretics, three were taking angiotensin-converting enzyme inhibitors and three were on β-blockers. Twenty millilitres of blood was transferred to chilled tubes containing 500 i.u./ml aprotinin (TrasyloÌ„, Bayer, Newbury, Berks, U.K.) and EDTA (1.5 mg/ml). After centrifugation, plasma was stored at −70 °C until assay. All samples were analysed within 2 months of venesection.

Plasma specimens were defrosted and 1 ml was acidified with an equal volume of 1% TFA. After centrifugation, the supernatant was loaded on to C₁₈ extraction columns. After two washes (3 ml each) with 0.1% TFA, the peptides were eluted with 2 ml of 0.1% TFA containing 60% acetonitrile. The eluates were then dried in a centrifugal evaporator. The dried eluates were reconstituted in 1 ml of the ILMA buffer (see below) containing 0.1% Triton X-100 and assayed immediately.

ILMA for NT-proBNP
The ILMA buffer consisted of 1.5 mmol/l NaH₂PO₄, 8 mmol/l Na₃HPO₄, 140 mmol/l NaCl, 1 mmol/l EDTA, 1 g/l BSA and 0.1 g/l azide. Wash buffer was composed of 1.5 mmol/l NaH₂PO₄, 8 mmol/l Na₃HPO₄, 140 mmol/l NaCl, 0.5 g/l Tween 20, 1 g/l gelatin and 0.1 g/l azide.

On day 1 of the assay, 100 μl of assay buffer containing 20 ng of the antibodies G172 or G185 was pipetted into tubes and incubated overnight at 4 °C with 100 μl of peptide standards in the range 1–2000 fmol per tube. All samples and standards were assayed in duplicate. One-hundred microlitres of assay buffer containing about 10⁶ relative light units (RLU) of the labelled peptide NT1 or NT2 was then added and tubes again incubated overnight at 4 °C. On day 3, 10 μl (10 μg) of paramagnetic particles coated with goat anti-rabbit IgG was added to tubes to recover the immunoprecipitates. The particles with attached immunoprecipitates were washed three times with the wash buffer described above (2 ml for each wash) and the particles recovered each time using a magnetized tube rack, allowing the wash solutions to drain adequately. After the last wash, 100 μl of distilled water was added to the tubes and the particles resuspended by vortexing the tubes. Readings of the chemiluminescence from the immunoprecipitates were then obtained on a Lumino portable luminometer (Stratec Electronic GMBH, Birkenfeld, Germany). In order to initiate chemiluminescence of the label [22,23], the first injection was 100 μl of 100 mmol/l HNO₃ containing 0.05% hydrogen peroxide, followed 4 s later by an injection of 100 μl of 250 mmol/l NaOH containing 0.25% cetyltriethylammonium bromide. The detergent optimized the light emission from the label. Chemiluminescence was measured over 2 s after the second injection and expressed as RLU. Standard curves were obtained and non-linear least squares fitting performed using an algorithm with a Rodbard 4 parameter equation.

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Echocardiography

Echocardiography was performed on all subjects using a Hewlett-Packard Sonos 1500 imaging system and recordings were on Panasonic Super VHS tapes. Left ventricular wall motion index (LVWMI, a regional measurement of LVD), which has been shown to be closely correlated to left ventricular ejection fraction by radionuclide cardiography and invasive ventriculography [21,22], was calculated using a nine-segment model originally described by Heger et al. [23]. The scale used for the LVWMI has been validated [24,25] and a linear correlation between LVWMI and left ventricular ejection fraction has been previously demonstrated [25]. LVWMI multiplied by 0.3 gives an estimate of left ventricular ejection fraction [21]. This nine-segment model for calculation of LVWMI was used in the large multi-centre TRACE study [26].

The scanning protocol consisted of obtaining the following views: parasternal long-axis sector of left ventricle, parasternal short axis at mitral valve level, parasternal short axis at papillary muscle level, apical four-chamber view and apical long-axis view. LVWMI was analysed while blind to the patient details.

Peptide blotting using tricine/SDS/polyacrylamide gels

Five to ten millilitres of plasma was acidified with an equal volume of 0.1% TFA, incubated on ice for 30 min and then centrifuged. The supernatants were then loaded on to C18 columns (containing 1 g of the sorbent material). After five washes with 4 ml each of 0.1% TFA, the peptides were eluted with 4 ml of 60% acetonitrile in 0.1% TFA. The samples were dried in a centrifugal evaporator.

Samples were then dissolved in 100 µl of gel sample buffer [consisting of 200 mmol/l Tris (pH 6.8), 20 g/l SDS, 0.4 g/l Coomassie Blue, 40% (by vol.) glycerol and 10 mmol/l dithiothreitol] and boiled for 5 min. They were then loaded to a 16.5% tricine/SDS/polyacrylamide gel. This consisted of a 16.5% resolving gel, a 10% spacer gel and a 5% stacking gel, made as described previously [27]. Gels were run at a constant voltage (90 V) over 16 h when the current fell from an initial 30 mA to 10 mA [27]. Coloured peptide markers were loaded to enable calibration of the observed bands. The peptides were then blotted to a nitrocellulose (0.2 µm) and blocked overnight in 1% dried milk powder in Tris-buffered saline (TBS, composed of 20 mmol/l Tris, 135 mmol/l NaCl) containing 0.1% Tween 20. Detection was achieved by incubating for 1 h at room temperature for 1 h. After 10 further washes the blot was developed using the Amersham enhanced chemiluminescence kit according to the instruction manual. Blots were exposed on to pre-flashed X-ray films to visualize the immuno-reactive bands.

Statistics

All results are expressed as medians (ranges) and comparisons were by the Mann–Whitney test. Spearman correlation analysis was performed on an Oxstat statistics package (Microsoft Corporation, Reading, U.K.). Two-tailed P values less than 0.05 were considered significant.

RESULTS

After labelling of the peptides NT1 and NT2 with the methyl acridinium ester, HPLC was essential to separate labelled from unreacted peptide. Peaks recovered at other acetonitrile concentrations on the gradient were unreactive with the antibodies. Although 10^4 RLU of label were added per tube, the non-specific binding was less than 1000 RLU using the paramagnetic particles to recover immunoprecipitates and with the washing protocol described above. The quantity of antibody added to tubes was titrated so that a non-saturating amount was added to each tube. Figure 1 illustrates the RLU measured in immunoprecipitates recovered with differing amounts of G172 or G185. On this basis, 20 ng was chosen as a suitable antibody amount.

Standard curves were constructed using known amounts of NT1 and NT2 and 20 ng of the antibodies

Figure 1 Relationship of the chemiluminescence of immunoprecipitates and different concentrations of antibodies G172 (for peptide NT1) and G185 (for peptide NT2) in the ILMA

Approximately 10^4 RLU of the labelled peptide NT1 or NT2 were added per tube at the beginning of the assay.
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Figure 2 Competitive binding curves for different amounts of the peptides NT1 and NT2 reacted with the specific antibodies G172 or G185 (20 ng per tube)
The chemiluminescence values were corrected to a value of 1 in the tubes with no added peptide.

G172 and G185. Subsequently 10⁶ RLU of label was added. The chemiluminescence of the recovered immunoprecipitates with differing amounts of added peptide standard is plotted in Figure 2. Increasing concentrations of each peptide displaced the label from the immunoprecipitates.

When pooled plasma from six normal controls was compared with that of six patients with NYHA class III or IV congestive heart failure, the mean level of plasma NT1 (measured with G172) was 12 fmol/ml compared with 18.8 fmol/ml respectively. These same pooled specimens were assayed for NT2 (with G185), and a level of 170 fmol/ml was recorded in normal controls compared with 1311 fmol/ml in congestive heart failure. Studies on NT1 in the extracts from pooled plasma in the presence of 0.1 % SDS (to denature the peptide) revealed no differences between the normal controls and the patients with congestive heart failure. Evaluation of NT1 as a diagnostic tool for LVD was abandoned in favour of NT2.

Further evaluation of the assay for NT2 indicated little cross-reactivity with other peptides. Cross-reactivity was < 0.1% for ANP, BNP, CNP or proBNP(22–46). Within-assay coefficients of variation for different concentrations of NT2 are reported in Table 1: different amounts of NT2 in buffer were extracted and then reconstituted for assay, using six aliquots at each concentration on the same day. Between-assay coefficients are also reported in Table 1, where the same preparations of NT2 utilized for the within-assay determinations were measured on five other occasions. The blank value of the assay (mean ± S.D., using water) was 1.3 ± 0.6 fmol/l.

Figure 3 illustrates a competitive binding curve for NT2 together with two plasma extracts from patients with congestive heart failure, diluted with ILMA buffer. The dilution curves are almost parallel within the working range of the assay (between about 5 and 200 fmol/tube).

NT2 levels were compared in plasma from the 12 patients with LVD and 12 normal subjects with no cardiovascular history and a normal LVWMI of 2. Plasma NT2 levels were elevated in patients with LVD [639 (386–911) fmol/ml] compared with normal controls [159 (120–245) fmol/ml, P < 0.001 by Mann–Whitney test]. The individual plasma NT2 values for the patients and controls are presented in Figure 4. In the population as a whole there was a correlation between LVWMI and NT2 levels (r = −0.74, P < 0.001).

The molecular mass of peptides exhibiting immunoreactivity to the antibody G185 was assessed using tricine/SDS/polyacrylamide gels to resolve the peptide mixtures present in extracts [27]. Figure 5 shows a blot where extracts from 10 ml of plasma from a normal control and 5 ml of plasma from a patient with congestive

Table 1 Assay coefficients of variation for ILMA of NT2

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DISCUSSION

There is much current interest in plasma BNP as a prognostic marker for cardiovascular events [5,9] and as a non-invasive diagnostic test for LVD [13–15]. By analogy with NT-ANP in the ANP system, NT-proBNP has been postulated to rise to a greater extent than BNP-32 in LVD [16,17]. Recently, NT-proBNP has been shown to be elevated after AMI and predicts 2-year survival [18]. Advantages of measuring NT-proBNP include the greater amounts present in the plasma of patients with congestive heart failure, potentially facilitating more accurate measurement. A non-radioactive assay would have advantages in ease of application for the purpose of LVD detection and monitoring.

We have devised a novel, sensitive and specific non-radioactive ILMA for the C-terminus of NT-proBNP (NT2). It was essential to separate the labelled peptides using HPLC since many peaks with the chemiluminescent label were essentially not immunoreactive. This may have been due to steric hindrance from the large chemiluminescent group added to the peptides. Although both assays are sensitive and capable of detecting the peptides at fmol concentrations, NT1 was not a suitable assay for further development as a diagnostic tool for LVD, presumably because the antibody did not react with this epitope on the intact endogenous NT-proBNP. Moreover, two recent studies using a commercial radio-immunoassay kit for proBNP(22–46) [28,29] also indicated that assay of this particular epitope was not useful in the assessment of LVD. In contrast, the assay of the C-terminus of NT-proBNP (termed NT2) revealed very significant differences in values between patients with LVD and normal controls and may be suitable for further development as a non-invasive and cost-effective diagnostic test. The levels in the normal controls that we have reported are significantly higher than that reported using a radio-immunoassay for the N-terminal region of NT-proBNP [17]. The reason for this is not obvious but may depend on varying immunoreactivity of the N- and C-terminal epitopes on the endogenous NT-proBNP compared with the free peptides used in the construction of standard curves (i.e. the antibody G185 may have a higher affinity for the C-terminus of endogenous NT-proBNP than the free unconjugated peptide used to construct the standard curves, so that the reported values of NT-proBNP are in equivalents of the C-terminal peptide fragment). Furthermore, it is not known at present whether the differences in the reported values for the levels of the N- and C-terminal epitopes of NT-proBNP reflect differences in stability to digestion by exopeptidases or endopeptidases in vivo. However, the ILMA we have described has the advantage of using non-radioactive techniques that do not require extensive laboratory radiation safety measures. The purified tracer is also stable in storage for over 6 months and is relatively

heart failure have been resolved on 16.5% tricine/SDS/polyacrylamide gels followed by detection of G185 immunoreactivity using enhanced chemiluminescence. One clear band of reactivity was present at a molecular mass of about 9.1 kDa. There was no reactivity at the level of the expected molecular mass of intact proBNP (or γ-BNP, 13.5 kDa). These findings were replicated on two other occasions. Thus, the antibody to the C-terminus of NT-proBNP detects the cleaved molecule rather than the intact proBNP peptide.

Figure 4 Plasma levels of NT2 in control subjects and patients with LVD
The medians are shown as solid bars and the values are significantly different ($P < 0.001$).

Figure 5 A 16.5% tricine/SDS/polyacrylamide gel of two plasma extracts [5 ml from one patient with LVD and 10 ml from one normal subject (CON)] resolved and blotted on to nitrocellulose and probed with the antibody G185
Peptide molecular mass standards are marked. One major band of immunoreactivity at 9.1 kDa is visible with no evidence of reactivity at the expected molecular mass of intact proBNP (13.5 kDa).
Inexpensive and easy to prepare. The between- and within-assay coefficients of variation are acceptable for a competitive assay. We also confirm that the assay of NT2 (an epitope at the C-terminal end of NT-proBNP) detects the presence of NT-proBNP rather than the intact proBNP (or \( \gamma \)-BNP) since the molecular mass of the immunoreactive material is about 9.1 kDa, very similar to the molecular mass of 8.6 kDa reported by another group [16–18].

In summary, we have demonstrated the possibility of using a chemiluminescent methyl acridinium ester derivative to label a small peptide and developed a competitive ILMA for NT-proBNP which may have potential in the diagnosis and monitoring of therapy in patients with LVD. More extensive studies on the usefulness of this assay are currently in progress.

ACKNOWLEDGMENTS

We thank the Leicester Royal Infirmary for supporting S.T., and the British Pharmacological Society for the research studentship to D.H. We are grateful to Dr Stuart Woodhead and Dr Ian Weeks for the advice on chemiluminescent labels. We also thank Ms Paulene Quinn and Sonja Jennings for excellent technical assistance.

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Received 20 August 1998/25 November 1998; accepted 8 December 1998