Coronary Heart Disease

B-Type Natriuretic Peptide Signal Peptide Circulates in Human Blood
Evaluation as a Potential Biomarker of Cardiac Ischemia

Maithri Siriwardena, MB, ChB; Torsten Kleffmann, PhD; Peter Ruygrok, MD; Vicky A. Cameron, PhD; Tim G. Yandle, PhD; M. Gary Nicholls, MD; A. Mark Richards, MD, PhD; Chris J. Pemberton, PhD

**Background**—The diagnosis of cardiac necrosis such as myocardial infarction can be difficult and relies on the use of circulating protein markers like troponin. However, there is a clear need to identify circulating, specific biomarkers that can detect cardiac ischemia without necrosis.

**Methods and Results**—Using specific immunoassay and tandem mass spectrometry, we show that a fragment derived from the signal peptide of B-type natriuretic peptide (BNPsp) not only is detectable in cytosolic extracts of explant human heart tissue but also is secreted from the heart into the circulation of healthy individuals. Furthermore, plasma levels of BNPsp in patients with documented acute ST-elevation myocardial infarction (n=25) rise to peak values (~3 times higher than the 99th percentile of the normal range) significantly earlier than the currently used biomarkers myoglobin, creatine kinase-MB, and troponin. Preliminary receiver-operating characteristic curve analysis comparing BNPsp concentrations in ST-elevation myocardial infarction patients and other patient groups was positive (area under the curve=0.97; P<0.001), suggesting that further, more rigorous studies in heterogeneous chest pain patient cohorts are warranted.

**Conclusion**—Our results demonstrate for the first time that BNPsp exists as a distinct entity in the human circulation and could serve as a new class of circulating biomarker with the potential to accelerate the clinical diagnosis of cardiac ischemia and myocardial infarction.

**Clinical Trial Registration**—URL: http://www.anzctr.org.au. Unique identifier: ACTRN12609000040268.

*(Circulation. 2010;122:255-264.)*

**Key Words:** acute coronary syndrome ■ biomarkers ■ ischemia ■ myocardial infarction ■ signal peptide
biological cells. Once this is complete, SPs have been generally thought to be degraded intracellularly. There is recent limited evidence, however, that some SPs or their fragments are released from the ER. SPs can be cleaved into N-terminal and C-terminal fragments, which are released into the cytosol of some cells. For example, an N-terminal fragment of preprolactin SP is released into the cytosol of bovine pituitary cells. Furthermore, the SP of mouse mammary tumor virus Rem protein is released from the ER membrane and accumulates in nucleoli. Not only are some SPs released from the ER, but thereafter they may have physiologically important functions beyond directing proteins into specific cellular pathways. One example relates to major histocompatibility complex I SP fragments, which have been shown to influence self-/non–self-recognition and natural killer cell activity at the cell surface. A second example is the N-terminus fragment of preprolactin SP, which interacts with cytosolic calmodulin to modulate intracellular pituitary Ca\(^{2+}\). Given this background, we hypothesized that a peptide derived from SP sequences not only may be present in the cell but also could enter the circulation. To this end, we assessed whether the SP sequence of B-type natriuretic peptide (BNP), a peptide hormone primarily released from the cardiac ventricles in response to cardiac wall stretch (strictly transmural pressure) and used as diagnostic plasma marker for suspected cardiac failure, could yield a circulating peptide.

### Methods

#### Chemicals

Synthetic human BNPsp (17-26), (Tyr)BNPsp (17-26), and (Cys)BNPsp (17-26) were synthesized by Mimotopes (Melbourne, Australia) and were confirmed as >95% pure by mass spectrometry (MS). All other synthetic peptides were purchased from the Peptide Institute (Osaka, Japan) or Sigma-Aldrich (St. Louis, Mo).

#### BNPsp (17-26) Assay Development

Specific antibodies to BNPsp (17-26) for use in immunoassay were developed according our previous protocols. Briefly, synthetic BNPsp (17-26) coupled to bovine BSA was injected subcutaneously into rabbits for immunization, and the primary antiserum was affinity purified. The cross-reactivity of BNPsp antiserum with other endogenous peptides was negligible (Table I in the online-only Data Supplement), and antiserum solutions were diluted in radioimmunoassay buffer. The assay incubate consisted of 100 µL extracted sample or standard [0 to 640 pmol/L of BNPsp (17-26) peptide] combined with 100 µL antiserum H13-3 and 100 µL iodinated (Tyr)BNPsp (17-26) (4000 to 6000 cpm). Tubes were incubated for 24 hours at 4°C, and then free BNPsp and bound BNPsp were separated by solid-phase second-antibody method (donkey anti-rabbit Sac-Cel, Immunodiagnostic Systems, Boldon, UK). Sac-Cel (1 mL) diluted in 5% dextran solution (final Sac-Cel concentration, 5%) was added to each tube; the solution was vortexed and incubated at room temperature for 30 minutes. Tubes were centrifuged at 2800g for 10 minutes at 20°C and decanted, with the pellet counted in a Gammamaster (LKB, Uppsala, Sweden). Assessment of hemoysis indicated that BNPsp immunoreactivity in assay was not altered up to a hemoglobin concentration of 8 g/L. Assessment of lipolysis indicated that BNPsp immunoreactivity was not altered by plasma lipid content up to 6g/L.

### Human Plasma Sample Collection

Human plasma samples were obtained from 6 patient groups (healthy volunteers and patients with cardiac catheterization, ST-elevation myocardial infarction [STEMI], chronic renal failure, congestive heart failure, and thyroid disease; see Table II in the online-only Data Supplement), and cardiac tissue samples obtained from those undergoing heart transplant surgery were procured in accordance with ethics protocols approved by local ethics committees (Canterbury, Auckland) of the Ministry of Health, New Zealand. Explant cardiac tissue donations were obtained from 10 patients enrolled in the Green Lane Hospital (Auckland, New Zealand) cardiac transplant program. Written consent was obtained before surgery in each case. Atrium and ventricular tissue samples (~10 g of each) were collected from explant hearts, washed in cold saline, immediately frozen at ~80°C, and stored until tissue extraction and analysis. Extracts of human plasma were prepared for the measurement of BNPsp immunoreactivity with solid-phase C18 cartridges, and cardiac tissue extracts from explant human hearts were prepared as previously reported. All participants gave informed consent before recruitment, and all investigations conform to the principles of the Declaration of Helsinki.

#### High-Performance Liquid Chromatography

Plasma and cardiac tissue extracts were dried under air, reconstituted in 60% acetonitrile/0.1% trifluoroacetic acid, and subjected to size-exclusion high-performance liquid chromatography (HPLC) on a Superdex G75 Superose column (Pharmacia Biotech, Uppsala, Sweden) followed by reverse-phase (RP) HPLC. Fractions were collected as previously described, dried, reconstituted, and subjected to BNPsp immunoassay.

#### MS Analysis of BNPsp

Endogenous human BNPsp (17-26) that was immunopurified from plasma was analyzed by matrix-assisted laser desorption/ionization time of flight (online-only Data Supplement). All MS spectra were acquired in positive-ion mode with 800 to 1000 laser pulses per sample spot. A maximum of 6 precursor ions of each sample spot were selected for MS/MS collision-induced fragmentation analysis. Modifications of endogenous BNPsp were analyzed by liquid chromatography multistage mass spectrometry (LC-MS\(^3\)) (LTQ-OrbitrapXL MS, Thermo Scientific, San Jose, Calif). Eluting peptides were monitored by a full mass scan using the linear ion trap in a mass range from m/z 400 to 1400. The predicted m/z value of the doubly charged peptide was selected as the exclusive precursor mass triggering subsequent scan events.

#### Statistics

Results are presented as mean±SD. Comparison of means was carried out with paired, 2-tailed Student t test when appropriate. Statistical differences between various biomarker peak times were determined by Wilcoxon signed-rank test. Assessment of multiple markers within individuals and regional NT-proBNP/BNPsp measurements from multiple regional venous sites was carried out with a nonparametric Friedman test followed by Tukey multiple-comparisons test with ranked sums. Analysis of data from independent samples was carried with the Kruskal-Wallis test followed by the Dunn method for multiple comparisons. Relational analyses of plasma hormone concentrations using Spearman rank-order correlation testing and receiver-operating characteristic curve analysis were carried out with SPSS version.
In all analyses, a value of $P < 0.05$ was considered significant.

**Results**

**Identification of Endogenous BNPsp Immunoreactivity in Human Plasma**

BNPsp contains 26 amino acids (Figure 1A). We generated an antibody (H13–3) directed toward the C-terminal 10 amino acids of BNPsp, i.e., BNPsp (17-26), and used it to establish a sensitive, specific radioimmunoassay (Figure 1B). This assay had a mean zero binding of $29 \pm 1\%$, sample detection limit of $5.0 \pm 0.6$ pmol/L, ED$_{50}$ of $161 \pm 8$ pmol/L, and a working range of 80 to 320 pmol/L in which the intra-assay coefficient of variation was 5.4%. Interassay coefficients of variation were 13.6% at 130 pmol/L and $\approx 12.8\%$ at 44 pmol/L. Cross-reactivity assessment of the antisera showed no detectable interference with other relevant peptides or with medications commonly used in cardiovascular disorders (Table I in the online-only Data Supplement). Initial assessment confirmed that BNPsp immunoreactivity was present in human plasma from peripheral blood collected into Na$_3$ EDTA and that it diluted in parallel with the synthetic standard curve (Figure 1B). The level of immunoreactive BNPsp present in normal human venous plasma obtained from 125 healthy volunteers was $14.1 \pm 4.7$ pmol/L (range, 7 to 25 pmol/L); immunoreactivity was detected in every sample. Correlation analysis of plasma BNPsp with concomitant NT-proBNP

![Figure 1](image_url)

**Figure 1.** Identification of BNPsp in human plasma. A, Epitope design for generation of H13–3 antiserum. Human preproBNP is 134 amino acids, the first 26 amino acids of which represent the SP. We generated a polyclonal antiserum to the carboxyl terminus amino acids 17 to 26 of the SP (single-letter amino acid notation shown). B, Representative BNPsp immunoassay curve showing parallel dilution of extracted human cardiac coronary sinus venous plasma (red line) and aortic root arterial plasma (green line) samples compared with synthetic BNPsp (17-26) peptide standard curve (black line). C, Concentrations of immunoreactive BNPsp in normal, healthy volunteer plasma samples showed a weak but statistically significant ($r = -0.23$, $P < 0.01$ by Spearman rank-order analysis) negative correlation with age. D, Regional vascular concentrations of NT-proBNP (top) and immunoreactive BNPsp (bottom) in 50 patients undergoing clinically indicated cardiac catheterization. Vascular regions are indicated as follows: FA1 = initial femoral artery sample, FV = femoral vein time matched to FA1, RV = renal vein, HV = hepatic vein, IVC = inferior vena cava, JUG = jugular vein, CS = cardiac coronary sinus, PA = pulmonary artery, FA2 = second (exit) femoral artery sample.
Figure 2. (Legend on next page).
levels, gender, and body mass index revealed no significant association, but there was a weak, although statistically significant, inverse correlation with age ($r=-0.23$, $P<0.01$; Figure 1C).

**Immunoreactive BNPsp Is Released From the Human Heart Into the Circulation**

Given that BNP is predominantly a cardiac peptide, we anticipated that like its congener NT-proBNP, immunoreactive BNPsp levels would be higher in blood draining the heart (ie, in cardiac coronary sinus samples) than in blood supplying the heart (ie, arterial samples). Indeed, coronary sinus plasma contained significantly higher concentrations of immunoreactive BNPsp than simultaneously drawn arterial plasma samples ($18.9\pm3.6$ versus $15.9\pm3.7$ pmol/L; $P<0.00001$; $n=50$; Figure 1D). Statistically significant differences in immunoreactive BNPsp concentrations were also observed across the head and neck, kidney, and lower limb (Figure 1D). Immunoreactive BNPsp appeared to be cleared across the liver ($P<0.05$). In contrast, NT-proBNP levels were clearly and solely elevated in cardiac coronary sinus plasma ($117.8\pm117.3$ versus $84.9\pm89.7$ pmol/L, coronary sinus versus arterial respectively; $P<0.00001$; $n=50$), and there was evidence of renal clearance of NT-proBNP ($P<0.001$; Figure 1D).

**Stability of Endogenous BNPsp in Human Plasma**

The stability of endogenous BNPsp immunoreactivity in blood and plasma and during its subsequent extraction is an important analytic prerequisite. The addition of aprotinin (a serine protease inhibitor) to venous collection tubes containing Na$_3$EDTA had no effect on measured BNPsp levels in venous blood collected from 5 patients undergoing clinically indicated diagnostic cardiac catheterization (Figure 1 in the online-only Data Supplement). However, whole blood collected into Na$_3$ EDTA and stored at room temperature for 24 hours before centrifugation gave plasma immunoreactive BNPsp levels $\approx75\%$ higher than in samples centrifuged immediately after venesection. This effect was completely blocked by storing whole blood at 4°C. The observed extraction efficiency of synthetic BNPsp (17-26) from plasma was $\approx70\%$.

**MS Characterization of BNPsp (17-26) and Modified BNPsp (17-26) in Human Plasma**

Given that immunoreactive BNPsp concentrations in plasma were elevated soon after the onset of symptoms in patients with STEMI (see below), we collected 30 mL peripheral venous blood from each of the 50 patients (cumulative volume, 1.5 L) who presented to Christchurch Hospital with STEMI within 4 hours of symptom onset. After solid-phase extraction and immunooaffinity purification, separation of purified immunoreactive BNPsp by RP-HPLC revealed 3 major components, one of which eluted close to a synthetic BNPsp (17-26) marker (Figure 2A). Analysis of the BNPsp plasma components by matrix-assisted laser desorption/ionization tandem time-of-flight MS and manual interpretation of collision-induced dissociation fragment spectra revealed a common core sequence of LHLAFLGGRS in all detected BNPsp (17-26) derivatives (Figure IIA and IIB in the online-only Data Supplement). The alternate elution times of this core sequence were found to be related to differential modifications of the peptide (Figure 2C), ranging in weight from 28 to 151 Da. In all collision-induced dissociation MS/MS spectra of modified peptides, only the detectable b ions (product ion of an amide bond cleavage containing the N-terminus) were shifted, whereas y ions (product ion of an amide bond cleavage containing the C terminus) from $y_2-y_9$ were constant (Figure IIC in the online-only Data Supplement), indicating that the modification resides on the N-terminal leucine. To gain further insights into the nature of some of these modifications, we conducted high-resolution LC-MS$^3$ experiments on collision-induced dissociation derived b$_2$ ions of modified BNPsp (17-26) using the high-energy collision cell of a LTQ-OrbitrapXL MS. The histidine immonium ion ([m/z] 110.07127) gave the strongest signal in all MS$^3$ spectra, confirming that the histidine side chain was unmodified. Under high-energy collision conditions for MS$^3$ of the b$_2$ ion, the most abundant peak carrying the modification was identified as an a$_1$-type ion, ie, the leucine immonium ion plus the mass adduct. In some cases, a leucine immonium ion ([m/z] 86.09643) was detectable in addition to the a$_1$ ion, indicating that in these cases the leucine side chain was not involved in the modification. Therefore, an N-terminal modification is most likely. Elemental composition analysis on either the MS$^2$ b$_2$ ions or MS$^3$ a$_1$ ions with a mass error of $<1$ mmu revealed clear chemical formulas of CO, C$_2$H$_4$O$_2$, C$_3$H$_4$O$_2$, and C$_3$H$_6$O$_2$ for the mass adducts relative to the unmodified peptide of 28, 58, 70, and 72 Da, respectively (Table III in the online-only Data Supplement). The elemental compositions of these detected modifications are consistent with formylation (CO) and modifications of amino groups by glyoxal (C$_2$H$_4$O$_2$) and methylglyoxal (C$_3$H$_9$O$_2$). Taken together, these results confirmed that our immunoassay detects authentic endogenous BNPsp (17-26) and the modified forms of BNPsp in human cardiac tissue and blood.

**Cardiac Tissue Levels and Molecular Forms of Immunoreactive BNPsp**

Atrial and ventricular extracts from explant cardiac tissue contained immunoreactive BNPsp, the concentrations of which, however, were much lower than corresponding BNP and NT-proBNP levels (Figure 3A). Average atrial BNPsp concentrations ($1.74\pm0.85$ pmol/g) were significantly higher than ventricular BNPsp concentrations ($1.09\pm0.41$ pmol/g; $P<0.05$; ratio, 1.5:1), but the atrial/ventricular ratio for BNPsp...
and NT-proBNP was much higher, \( \approx 10:1 \) for both. No statistical correlation was found between ventricular BNPsp and NT-proBNP concentrations \( (r = -0.55, P = 0.09; \text{Figure 3B}) \). RP-HPLC and size-exclusion HPLC analyses confirmed that cardiac tissue extracts contain low-molecular-weight BNPsp species \( \approx 1 \text{kDa} \) in size (Figure 3C).

**BNPsp Is an Early Rising Biomarker of Cardiac Ischemia**

Having established that immunoreactive BNPsp is present in the human heart and peripheral circulation and that the cardiac coronary sinus is a source of the circulating peptide, we sought to determine the potential utility of circulating BNPsp as a diagnostic biomarker of acute cardiac ischemia resulting in myocardial infarction. In patients with documented STEMI whose symptom onset was \( <4 \text{ hours} \) before presentation to Christchurch Hospital, peripheral venous plasma concentrations of BNPsp were markedly elevated 4 to 5 hours after symptom onset but fell thereafter, returning to the normal range within 10 hours (Figure 4A). Average peak levels were \( \approx 6 \)-fold higher than the average observed in normal health and \( >3 \)-fold beyond the 99th percentile upper limit of normal (25 pmol/L). Of the 25 STEMI patients, 19 had TnI \( >0.03 \mu \text{g/L} \) at presentation, and 23 had TnI \( >0.03 \mu \text{g/L} \) at 1 hour after presentation. Peak levels of BNPsp tended to correlate positively with simultaneously measured
CK-MB ($r=0.28$, $P=0.128$) and myoglobin ($r=0.31$, $P=0.105$). There was no significant association between peak BNPsp and peak Tnl nor between peak BNPsp and peak NT-proBNP. The time to peak BNPsp after symptom onset was significantly earlier than that for myoglobin ($P<0.05$) and CK-MB, troponin I, and NT-proBNP (all $P<0.01$; Figure 4A). In contrast to BNPsp, plasma NT-proBNP rose steadily over a much longer time course, reaching peak levels $\approx 76$ hours after symptom onset (Figure 4A).

Average venous BNPsp levels in patients with chronic renal disease were significantly higher than the mean in healthy volunteers but were nevertheless still within the normal range (Figure 4B). Patients with hyperthyroidism or hypothyroidism and congestive heart failure had mean BNPsp levels no different from normal subjects. Receiver-operating characteristic curve analysis of BNPsp levels 5 hours after chest pain onset to detect STEMI (n=25) compared with normal control subjects (n=125) and patients with thyroid disease (n=11), chronic renal failure (n=34), and congestive heart failure (n=10) generated an area under the curve of 0.97 ($P<0.001$), a sensitivity of 88%, and a specificity of 92% at 25 pmol/L (99th percentile of the upper limit of the normal range; Figure 4C). Receiver-operating characteristic curve analysis of Tnl at the same time point gave an area under the curve of 0.99 ($P<0.001$), a sensitivity of 96%, and a specificity of 95% with a 99th percentile cutoff of 0.03 µg/L.

Discussion

Our results are the first demonstration of a defined signal sequence as a distinct, separate entity within the circulation in humans. Whereas Christofferson et al$^{18}$ reported that the SP of apolipoprotein M is retained along with the entire apolipoprotein M protein in the circulation of humans and that this
uncleaved SP helps prevent renal filtration of apolipoprotein M through hydrophobic association with circulating lipoproteins, they did not detect the apolipoprotein M SP as a distinct, separate entity. We found no evidence on immunoassay or HPLC for the SP fragment BNPs (17-26) in human plasma to be associated with proBNP (1-108), ie, as a component of preproBNP (1-134). Nevertheless, we cannot exclude the existence of such an association because our BNPs (17-26) assay is designed to detect free carboxyl terminal residues in position 26 of the SP, which requires cleavage of proBNP (1-108). Future work with assays directed toward the amino terminal region of preproBNP (1-134) is needed to definitively answer whether it also exists in the circulation.

Study of the regional plasma levels of NT-proBNP revealed only 1 site of clear secretion, namely the cardiac coronary sinus. This observation underscores the cardiovascular utility of NT-proBNP measurement and suggests that the heart is the only significant contributor to circulating NT-proBNP levels. In contrast, we observed significant elevations in circulating BNPs levels across the heart, head/neck, kidney, and lower limbs. That the heart should be a significant contributor to circulating BNPs is understandable; the other putative sites of secretion are less so. However, it should be noted that in humans, both the pituitary and kidney contain significant amounts of preproBNP RNA transcript, at levels only 10 to 100 times less than cardiac levels. This pattern of human BNP tissue expression contrasts with results from animal studies that report cardiac BNP expression to be 1000 times higher than any other organ. Thus, it is conceivable that the head and kidney could contribute to BNPs secretion in humans. However, it is unclear as to why this should be apparent for BNPs but not NT-proBNP. Additional studies are required to address this issue, along with how the lower limbs might potentially contribute to circulating BNPs levels.

The generation of BNPs (17-26) from the 26-amino acid SP is consistent with previous reports that have outlined a 2-step proteolysis of SPs in the ER membrane. Thus, in the case of preproBNP, cleavage of the SP from the propeptide by signal peptidease followed by further cleavage at the midregion hydrophobic core by SP peptidase generates the C-terminus peptide beginning with residue Leu. However, high-resolution MS on MS2 and MS3 fragment ions suggested that the N terminus of Leu carried differential modifications ranging in molecular weight from 28 to 151 Da. Elemental composition analyses of low-mass ions revealed clear chemical formulas for some of these modifications that are identical to the chemical compositions of modifications of \( \epsilon \)-amino groups of lysines by glyoxal and methylglyoxal forming \( N^2 \)-carboxymethyl-lysine and \( N^\epsilon \)-carboxyethyl-lysine, respectively. Both modifications can be either a product of the decomposition of protein glycations referred to as advanced glycation end products or a direct reaction of amino groups with reactive carbonyl and dicarbonyl compounds generated by oxidative degradation of sugars and lipid peroxidation. An increased level of advanced glycation end products and similar nonenzymatic protein modifications is associated with conditions such as diabetes mellitus, atherosclerosis, inflammatory conditions, and cardiac ischemia/reperfusion. It has been shown that oxidative stress resulting from ischemic injuries in rat hearts and elevated levels of peroxynitrite can be a source of rapid generation of methylglyoxal and glyoxal and nonenzymatic protein modifications.

The majority of immunopurified BNPs (17-26) from STEMI patients was N-terminally modified. Nonenzymatic protein modifications by compounds generated in the process of oxidative stress may result in a very complex profile of different modifications as we have observed for BNPs (17-26). Further analyses of the BNPs (17-26) modifications are required to properly characterize the origin of nonenzymatic peptide modifications of BNPs (17-26) in plasma.

The rise in plasma BNPs in patients with documented STEMI occurred significantly earlier than all other current markers of acute myocardial infarction (myoglobin, CK-MB, TnI). Our preliminary receiver-operating characteristic curve analysis suggests that plasma BNPs has potential in the diagnosis of acute coronary syndromes, although it is obviously limited by the fact that it comprised only 25 STEMI patients. Further study in a larger “all comers” acute coronary syndromes cohort that includes potential false-positive/negative patients is clearly required to determine BNPs assay specificity in cardiac ischemia and acute coronary syndromes. Although peak circulating BNPs levels did not significantly associate with peak CK-MB, myoglobin, or TnI levels in our STEMI cohort, the sample size was small and hence additional study is needed. Why circulating BNPs levels should peak so early after STEMI is unclear but may relate to the comparatively smaller weight of this signal sequence (Mr 1070 versus Mr 15 000 to 40 000 for the other 3 markers of acute myocardial infarction noted above). Consistent with this line of reasoning is the observation that BNPs was cleared much more quickly from the circulation than myoglobin, TnI, and CK-MB; its approximated clearance time was 15 minutes. Such a pathophysiological profile suggests that BNPs may be located within the myocardial cells adjacent to the outer membrane or that intracellular stores of the peptide are subject to a rapid release mechanism on ischemic challenge.

The relative concentrations of BNPs (17-26) we observed in myocardium and plasma raise intriguing questions about secretion of the peptide. The facts that first there was no significant relationship between cardiac BNPs and BNPs/NT-proBNPs concentrations and second the atrial concentrations of BNPs were only 1.7 times higher than ventricular BNPs (compared with an atrial/ventricular ratio of \( \approx 10 \) times for BNPs and NT-proBNPs) suggest that the storage and secretion of BNPs differ substantially from that for BNPs/NT-proBNPs. It is currently considered that secretion of BNPs and NT-proBNPs occurs by the regulated, storage granule-based pathway in cardiac atria and by constitutive secretion in the ventricles. Release of these storage granules is dictated largely by alterations in cardiac transmural pressure but contributed to by factors such as local myocardial hypoxia and pH, as well as hormones like endothelin-1 and angiotensin II. However, mechanisms governing BNPs levels in the circulation of normal, healthy individuals remain unknown.
unknown. It is known that the SP sequence of a preproprotein encodes for cellular secretion or storage of that protein, but not all secreted proteins in the circulation meet this “classic” criteria because they have no discernible SP motif. This is true for established markers of cardiac necrosis such as CK-MB and myoglobin, both of which appear in the circulation in normal health. How “nonclassic” secretion pathways such as membrane blebbing or shedding, endosomally recycling, or active membrane transportation might relate to BNPsp secretion is an obvious target for future in vitro studies. Furthermore, the role of the ER and the unfolded-protein response (a cytotoxic mechanism in the face of impending cellular ischemia) in channeling or controlling BNPsp secretion also needs to be considered.

Conclusions
We provide the unique observation that BNPsp (17-26) exists in the circulation of healthy individuals and could be used as a marker of cardiac ischemia/infarction. Further studies in a prospective patient group with recent onset of chest pain with multiple confounding diagnoses are now required to determine the diagnostic specificity of BNPsp in cardiac ischemia, especially compared with other putative markers of ischemia such as ischemia-modified albumin, free fatty acids, glycogen phosphorylase isoenzyme BB, and high-sensitivity troponin.

Acknowledgments
We thank the technical staff of Canterbury Health Laboratories and Endolab, Christchurch Hospital, New Zealand, for assistance with biomarker assays and the research nursing staff of the Cardioendocrine Research Group for assistance with patient blood sampling.

Sources of Funding
This work was supported by the Health Research Council of New Zealand (grant 07/114) and the National Heart Foundation of New Zealand (grant 1351). Dr Pemberton is the recipient of a Health Research Council of New Zealand Sir Charles Hercus Research Fellowship, and Dr Richards holds the National Heart Foundation of New Zealand Professorial Chair in Cardiovascular Studies.

Disclosures
The University of Otago, Christchurch, New Zealand, has filed a patent application on the composition and diagnostic/prognostic use of BNPsp measurement in acute cardiovascular disorders. Drs Pemberton, Richards, Nicholls, and Yandle are listed as inventors on this application. The other authors report no conflicts.

References
CLINICAL PERSPECTIVE

The clinical diagnosis of acute coronary syndromes relies heavily on circulating diagnostic biomarkers such as troponin. However, delays in detectable changes in circulating troponin, combined with their absence in ischemia short of infarction, result in clinical uncertainty in a significant number of patients presenting with suspected acute coronary syndromes. Thus, identification of novel biomarkers that may provide early information on acute myocardial infarction and cardiac ischemia is of major importance. We provide here the identification of a novel potential biomarker of acute coronary syndromes, namely a peptide fragment derived from the signal peptide region of B-type natriuretic peptide (BNPsp). BNPsp is present as a distinct peptide in explant human cardiac tissue and is secreted into the circulation in normal health. Furthermore, detectable elevations in BNPsp were observed in ST-elevation myocardial infarction patients significantly earlier than myoglobin, creatine kinase-MB, and troponin. BNPsp thus presents as a novel class of potential biomarker in acute coronary syndromes, and further studies to determine its assay specificity and diagnostic potential in the complete spectrum of acute coronary syndromes are clearly warranted.
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_Circulation_. 2010;122:255-264; originally published online July 6, 2010;
doi: 10.1161/CIRCULATIONAHA.109.909937
_Circulation_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7322. Online ISSN: 1524-4539

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circ.ahajournals.org/content/122/3/255

Data Supplement (unedited) at:
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SUPPLEMENTAL MATERIAL

MS ID#: CIRCULATIONAHA/2009/909937

MS TITLE: BNP SIGNAL PEPTIDE CIRCULATES IN HUMAN BLOOD: EVALUATION AS A POTENTIAL BIOMARKER OF CARDIAC ISCHEMIA

AUTHORS: Maithri Siriwardena, MB,ChB; Torsten Kleffmann, PhD; Peter Ruygrok, MD; Vicky A. Cameron, PhD; Tim G. Yandle, PhD; M. Gary Nicholls, MD; A. Mark Richards, MD/PhD; Chris J. Pemberton, PhD
Supplemental Methods

Generation of anti-human BNP-SP antisera
Synthetic (Cys)BNPsp(17-26) was coupled to maleimide treated/ECMS derivatised bovine serum albumin (BSA) and injected intradermally into two New Zealand White rabbits over 5–6 sites. Rabbits were bled 11–13 days after injection and the procedure repeated 4–6 weekly until adequate antiserum titres were obtained.

Preparation of 125I-radiolabelled human (Tyr)BNPsp(17-26)
Human (Tyr)BNPsp(17-26) (2.5 µg) was iodinated using 0.5 mCi Na-125I in the presence of 5 µg chloramine T in 5 µl of 0.5 M phosphate buffer, pH 7.3 for 15-20 s. The reaction was stopped by addition of 50 µg cysteine HCl in a further 5 µl of phosphate buffer. The resulting iodinate was loaded onto a 10 cm RP300 Brownlee HPLC column (Applied Biosystems, San Jose, CA) and pure (125I-Tyr)BNPsp(17-26) eluted with a gradient of 0% to 60% acetonitrile in 0.1% trifluoroacetic acid (TFA) over 15 min at a flow rate of 1 ml/min.

Human plasma sample collection
Human plasma samples obtained from patient groups i) to vi) and cardiac tissue samples obtained from those undergoing heart transplant surgery were procured in accordance with ethical protocols approved by Ethics Committees (Canterbury, Auckland) of the Ministry of Health, New Zealand. All participants gave informed consent before recruitment and all investigations conform to the principles of the Declaration of
Helsinki. Demographic data of each patient group from which plasma samples were drawn is provided in SD Table 2. Plasma samples were drawn from the following study groups: i) healthy volunteers with no evidence of cardiovascular, endocrine or psychiatric illness (n=125), ii) patients undergoing clinically indicated cardiac catheterisation (n=50), iii) patients with documented ST-elevation myocardial infarction (n=25), iv) patients with chronic renal failure (n=34), v) patients with known thyroid disease (n=11) and vi) patients with congestive heart failure (n=10).

All patients had blood drawn into chilled Vacutainer tubes containing 1.8mg/ml Na$_3$-EDTA (Becton-Dickinson). For groups i), iv) and v), single samples were taken from a forearm vein in the morning, immediately centrifuged to prepare plasma and stored at -80°C. Patients in group ii) were catheterised via the left femoral artery and had blood drawn from regional vascular sites (femoral vein, renal vein, hepatic vein, inferior vena cava, jugular vein, cardiac coronary sinus and pulmonary artery). Entry and exit femoral arterial samples were taken for comparison. STEMI patients in group iii) (registered at the Australia New Zealand Clinical Trials Registry [Http://www.anzctr.org.au], trial number ACTRN12609000040268) had forearm venous samples taken at hospital presentation at the following times after chest pain onset: 4hr, 4.5hr, 5hr, 6hr, 8hr, 12hr, 16hr, 28hr, 52hr and 76hr. Blood samples were immediately stored at 4°C, centrifuged for plasma within 4hrs of drawing and stored at -80°C, based on the collection parameter results provided in SD Figure 1. Patients in group vi) had blood samples drawn at hospital presentation, at 24-48 hours after presentation and at hospital discharge.
Collection stability of endogenous immunoreactive BNPsp in human blood

In order to assess the effects of collection variables and potential confounding parameters upon measured concentrations of immunoreactive BNPsp, 36mls of jugular venous blood was collected from each of 5 patients undergoing cardiac catheterisation into two groups of chilled Na\(^3\)-EDTA tubes. Group one tubes contained 1.8mg/ml Na\(^3\)-EDTA alone whereas group 2 tubes contained Na\(^3\)-EDTA + 100KIU/ml aprotinin inhibitor. Each group had two tubes, one of which was left at room temperature and one at 4°C for 0, 8 and 24 hrs. At each of these time points whole blood was centrifuged and plasma stored at -80°C prior to extraction. Thus, the potential effects of proteolytic inhibitor and storage temperature upon BNPsp collection were assessed.

High Performance Liquid Chromatography (HPLC)

Extracts were dried under air, reconstituted in 60% acetonitrile/0.1%TFA and subjected to size exclusion (SE) HPLC on a Superdex G75 Superose column (Pharmacia Biotech, Sweden), using an isocratic gradient of the above buffer at a flow rate of 0.25 ml/min. Fractions were collected at 2 min intervals and subjected to BNPsp immunoassay to establish immunoreactive molecular size. Fractions from SE-HPLC were then pooled, lyophilised, reconstituted in 0.1%TFA and eluted from a 5 µ reverse phase (RP) C18 Jupiter column (Phenomenex, Torrance, CA) with a gradient of 0%–60% acetonitrile over 60 min at a flow rate of 1 ml/min. Fractions were collected, dried, reconstituted and re-subjected to BNPsp immunoassay. RP-HPLC was calibrated with synthetic BNPsp(17-26) after plasma samples were run whereas SE-HPLC was calibrated with the following
markers: cytochrome C (Mr 12,000), aprotinin (Mr 6,000), CNP22 (Mr 2,000) and angiotensin II (Mr 1,000).

Purification and tandem MS/MS identification of endogenous circulating human BNPsp

BNPsp(17-26) immunoreactivity identified by coupled immunoassay/HPLC was purified from approximately 0.75 l of plasma drawn from 50 patients with documented acute STEMI. For each patient, 30 ml of peripheral venous blood was drawn into chilled tubes containing Na<sup>3</sup>-EDTA within 5 hrs of the onset of symptoms and 1 hr of hospital presentation. The samples were centrifuged at 4°C and plasma stored at -80°C until it was slowly thawed, extracted on C<sub>18</sub> cartridges and evaporated to dryness. Extracts were then concentrated by reconstitution in a minimal volume of immunoaffinity buffer (pH 7.4, SDMethds), centrifuged at 10,000g to pellet solid debris and then combined to a single solution. This solution was run under gravity at 4°C through an H13-3 anti-BNPsp(17-26) IgG coupled AminoLink™ gel prepared according to the manufacturers instructions (Pierce Biotechnology, IL). The column was then washed with a 5x volume of immunoaffinity buffer (pH 7.4) and eluted with 0.1M glycine, pH 2.5. Elution fractions containing immunoreactive BNPsp were submitted to RP-HPLC purification. BNPsp immunoreactive fractions identified after immunoaffinity/RP-HPLC purification were analysed for the presence of BNPsp(17-26) sequence using MALDI tandem time-of-flight mass spectrometry. Peptide modifications were further analysed by LC-MS<sup>3</sup>-experiments on a LTQ-OrbitrapXL mass spectrometer (Thermo Scientific, San Jose, CA).
MALDI tandem time-of-flight mass spectrometry of endogenous BNPsp immunoreactivity

RP-HPLC fractions containing immunopurified BNPsp were resuspended in 30% [v/v] acetonitrile and 0.1% [v/v] trifluoroacetic (TFA) acid in water. One microlitre of peptide solution was premixed with 2 µl of matrix (10 mg/ml \{\text{alpha}\}cyano-4-hydroxycinnamic acid dissolved in 65% [v/v] aqueous acetonitrile containing 0.1% TFA and 10 mM ammonium dihydrogen phosphate). 0.8 µl of sample/matrix mixture were spotted onto a MALDI sample plate (Opti-TOF 384 well plate, Applied Biosystems, MA) and air dried. Samples were analysed on a 4800 MALDI tandem Time-of-Flight Analyser (Applied Biosystems, MA). All MS spectra were acquired in positive-ion mode with 800–1000 laser pulses per sample spot. A maximum of six precursor ions of each sample spot were selected for MS/MS collision-induced fragmentation (CID) analysis. CID spectra were acquired with 2000–4000 laser pulses per sample spot using the 2 kV mode and air as the collision gas at a pressure of 1 x 10^{-7} torr.

Liquid Chromatography coupled LTQ-Orbitrap analysis of peptide modifications

Some of the unknown modifications of the core peptide sequence of BNPsp(17-26) were analyses by MS^3 experiments on a LTQ-OrbitrapXL mass spectrometer (Thermo Scientific, San Jose, CA) in-line coupled to an Ultimate 3000 nano-flow HPLC-system (Dionex Co,CA). Approx. 200 fmol of each peptide was loaded individually in 5ul of 1% [v/v] acetonitrile, 0.2% [v/v] formic acid in water onto an in-house packed nanospray emitter-tip column (75 µm ID fused silica tubing packed with C_{18} material on a length of 8-9 cm) and eluted by a gradient developed from 1% [v/v] acetonitrile, 0.2% [v/v] formic
acid to 80% [v/v] acetonitrile, 0.2% [v/v] formic acid in water over 15 min. at a flow rate of 200 nl/min. Eluting peptides were monitored by a full mass scan using the linear ion trap in a mass range from m/z 400-1400. The predicted m/z value of the doubly charged peptide was selected as the exclusive precursor mass triggering subsequent scan events. The following data dependent scan events were triggered when the selected precursor exceeded a peak intensity of >10000 counts: (i) An Orbitrap single ion monitoring of the selected precursor ion in a mass window of +/- 5 Da with a resolution of 60000 at m/z 400. (ii) A collision induced dissociation of the precursor ion in the linear ion trap at a normalised collision energy of 35% and fragment ion measurement in the Orbitrap analyser with a resolution (FWHM) of 60000 at m/z 400. (iii) If a product ion with the mass of the predicted b2 ion was detected high energy collision dissociation (HCD) in the HCD collision cell was performed on the b2-ion at normalised collision energy of 52 % using nitrogen as collision gas. MS3 fragment ions were measured in the Orbitrap analyser with a resolution (FWHM) of 100000 at m/z 400. For all Orbitrap measurements the lock mass option was enabled for internal calibration on m/z 445.120025 (Si(CH3)_2O)_6). The AGC targets were set to 2e5 charges for the ion trap full scans, 1e5 and two microscans for MSn in the ion trap and 2e5 for the Orbitrap analyser. Dynamic exclusion was disabled.

**MS Data Analysis**

MALDI TOF and LTQ Orbitrap CID spectra of the unmodified peptide were submitted to a Mascot ([http://www.matrixscience.com](http://www.matrixscience.com)) search against the UniProtKB/Swiss-Prot amino acid sequence database (downloaded April 2009) to confirm the identification of
the BNPsp(17-26) core sequence. No enzyme and no modification were selected. The allowed mass errors were 75 p.p.m for MALDI MS with 0.4 Da fragment ion tolerance or 10 p.p.m. for Orbitrap MS with 0.01 Da fragment ion tolerance. In spectra of modified BNPsp(17-26) the presence of the core sequence LHLAFLGGRS was confirmed by manual spectrum interpretation considering b- and y-type ions according to the Biemann nomenclature. Conclusions on the chemical composition and localisation of modification of BNPsp(17-26) were drawn on Orbitrap measurements of the b2-ion from the CID MS² spectrum or the a1-ion from the HCD MS³ spectrum. Elemental composition analysis of the mass adducts of BNPsp(17-26) were performed on Orbitrap measurements of either the CID MS² b2- or HCD MS³ a1-ion with a mass tolerance of less than 1 mmu using the Xcalibur software tools. The range of mass errors of CID MS² and HCD MS³ spectra were estimated based on the measurements of the b2-ion from unmodified peptide and leucine and histidine immonium ions in MS³ spectra. The calculated chemical formulae were confirmed by the comparison of the predicted and measured ¹⁵N and ¹³C isotope pattern of the b2-ions using the Xcalibur software tools.
**Supplemental Tables**

**Table 1.** Cross reactivity data of H13-3 rabbit anti-human BNPsp(17-26) antiserum. All peptides are human forms.

<table>
<thead>
<tr>
<th>Peptide/Drug</th>
<th>Cross reactivity with BNPsp(17-26) antiserum (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BNPsp(17-26)</td>
<td>100</td>
</tr>
<tr>
<td>BNPsp(1-10)</td>
<td>&lt;0.002</td>
</tr>
<tr>
<td>proBNP(1-13)</td>
<td>&lt;0.003</td>
</tr>
<tr>
<td>proBNP(1-76)</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>proANP(1-30)</td>
<td>&lt;0.009</td>
</tr>
<tr>
<td>ANP</td>
<td>&lt;0.008</td>
</tr>
<tr>
<td>BNP</td>
<td>&lt;0.009</td>
</tr>
<tr>
<td>Endothelin 1</td>
<td>&lt;0.006</td>
</tr>
<tr>
<td>Angiotensin II</td>
<td>&lt;0.003</td>
</tr>
<tr>
<td>Angiotensin(1-7)</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Urotensin II</td>
<td>&lt;0.003</td>
</tr>
<tr>
<td>CNP22</td>
<td>&lt;0.006</td>
</tr>
<tr>
<td>proCNP(1-15)</td>
<td>&lt;0.008</td>
</tr>
<tr>
<td>Adrenomedullin</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Urocortin I</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Urocortin II</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Clopidigrel</td>
<td>0</td>
</tr>
<tr>
<td>Morphine</td>
<td>0</td>
</tr>
<tr>
<td>Aspirin</td>
<td>0</td>
</tr>
</tbody>
</table>
Table 2. Demographic data (mean±SEM) for each of the six patient groups studied.

<table>
<thead>
<tr>
<th></th>
<th>Normal controls (n=125)</th>
<th>Catheterisation (n=50)</th>
<th>STEMI (n=25)</th>
<th>Thyroid disease (n=11)</th>
<th>Renal failure (n=34)</th>
<th>CHF (n=10)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Age (yrs)</strong></td>
<td>57.0±1.3</td>
<td>65.6±1.5</td>
<td>61.3±2.9</td>
<td>40.9±4.6</td>
<td>52.0±2.7</td>
<td>68.0±5.0</td>
</tr>
<tr>
<td><strong>Male</strong></td>
<td>77 (62%)</td>
<td>36 (72%)</td>
<td>19 (76%)</td>
<td>6 (55%)</td>
<td>21 (62%)</td>
<td>8 (80%)</td>
</tr>
<tr>
<td><strong>BMI (kg.m²)</strong></td>
<td>26.3±0.4</td>
<td>29.4±0.9</td>
<td>26.5±0.9</td>
<td>-</td>
<td>27.8±0.9</td>
<td>27.6±3.7</td>
</tr>
<tr>
<td><strong>Hypertension</strong></td>
<td>27 (21.6%)</td>
<td>32 (64%)</td>
<td>10 (40%)</td>
<td>1 (9%)</td>
<td>28 (82%)</td>
<td>4 (40%)</td>
</tr>
<tr>
<td><strong>Diabetes</strong></td>
<td>8 (6.4%)</td>
<td>11 (22%)</td>
<td>1 (4%)</td>
<td>-</td>
<td>3 (9%)</td>
<td>1 (10%)</td>
</tr>
<tr>
<td><strong>Hyperlipidemia</strong></td>
<td>25 (20.0%)</td>
<td>-</td>
<td>1 (4%)</td>
<td>1 (9%)</td>
<td>-</td>
<td>3 (30%)</td>
</tr>
<tr>
<td><strong>GFR</strong></td>
<td>-</td>
<td>61.9±3.5</td>
<td>-</td>
<td>76.0±11.9</td>
<td>29.0±2.7</td>
<td>70.0±5.3</td>
</tr>
<tr>
<td><strong>Thyroid disease</strong></td>
<td>7 (5.6%)</td>
<td>-</td>
<td>-</td>
<td>11 (100%)</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>
### Table 3: Mass spectrometric characterisation of BNPsp(17-26) modifications.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Peptide mass</th>
<th>#Mass adduct</th>
<th>m/z values of the most abundant HCD MS3 fragment ions</th>
<th><strong>Predicted formula of mass adduct relative to unmodified BNPsp(17-26).</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>TOF/TOF MS estimated mass error &lt; 30ppm</td>
<td>Orbitrap MS estimated mass error &lt; 2ppm</td>
<td>TOF analysis of precursor mass estimated mass error &gt; 30mmu</td>
<td>Orbitrap analysis of b2- ions estimated mass error &lt; 1mmu</td>
</tr>
<tr>
<td>38</td>
<td>1069.615</td>
<td>1069.60447</td>
<td>n/a</td>
<td>n/a</td>
</tr>
<tr>
<td>38</td>
<td>1220.617</td>
<td>-</td>
<td>151.002</td>
<td>-</td>
</tr>
<tr>
<td>39</td>
<td>1197.619</td>
<td>1197.60812</td>
<td>128.004</td>
<td>128.00426</td>
</tr>
<tr>
<td>41</td>
<td>1141.626</td>
<td>1141.62413</td>
<td>72.011</td>
<td>72.02083</td>
</tr>
<tr>
<td>41</td>
<td>1127.615</td>
<td>1127.60971</td>
<td>58.000</td>
<td>58.00536</td>
</tr>
<tr>
<td>41</td>
<td>1097.603</td>
<td>1097.63499</td>
<td>27.988</td>
<td>27.99471</td>
</tr>
<tr>
<td>42</td>
<td>1212.603</td>
<td>1212.61021</td>
<td>142.988</td>
<td>143.00383</td>
</tr>
<tr>
<td>43</td>
<td>1172.603</td>
<td>-</td>
<td>102.988</td>
<td>-</td>
</tr>
<tr>
<td>44</td>
<td>1139.596</td>
<td>1139.61143</td>
<td>69.981</td>
<td>70.00524</td>
</tr>
</tbody>
</table>

# The calculated mass adduct is based on either the mass difference between unmodified and modified BNPsp(17-26) after MALDI TOF analysis or on the mass shift between b2-ions after CID-fragmentation of unmodified and modified BNPsp(17-26) measured in the Orbitrap analyser.

** The predicted formulae of the mass adducts are based on an elemental composition analyses on either (b2) MS2 measurements of b2-ions after CID fragmentation or (a1) MS3 measurements of a1-ions after HCD fragmentation of b2-ions in the Orbitrap analyser.

The lock mass option for internal calibration on m/z 445.12003 was enabled for all Orbitrap measurements.
Supplemental Figures

**Figure 1.** Stability of endogenous immunoreactive BNPsp in whole blood samples drawn from the jugular vein of five patients undergoing cardiac catheterisation. Whole blood (5ml) was drawn into tubes containing the inhibitors shown and left for 0, 8 and 24hrs at room temperature (RT) or 4°C prior to centrifugation, storage at -80°C and subsequent immunoassay. Aprotinin had no effect over and above that of EDTA alone upon endogenous measured levels whereas storage at room temperature prior to centrifugation resulted in elevations in immunoreactive BNPsp.
Figure 2

A

endogenous BNPsp(17-26)

B

synthetic BNPsp(17-26)
Figure 2. MALDI TOF/TOF CID spectra of unmodified and modified BNPsp(17-26). A) Manual sequence interpretation of immunoaffinity purified endogenous BNPsp(17-26) ([M+H]\(^+\) of 1070.623) and B) synthetic BNPsp(17-26). Detected b-ions and y-ions conclusively identify the core amino acid sequence LHAFLLGR in both spectra as indicated. C) CID-spectra of some of the modified BNPsp(17-26) peptides. All spectra showed a constant y-ion series from \(y_2\)-\(y_9\) consistent with unmodified peptide confirming the core sequence of BNPsp(17-26) in all modified species. Detectable b-ions starting with \(b_2\) were shifted by the indicated mass adduct which suggests a modification of either the N-terminus or the two N-terminal amino acids leucine and histidine or a combination of different modifications.
Figure 3. Representative example for LTQ-Orbitrap analyses of BNPsp(17-26) modifications. A) Orbitrap measurement of CID fragments of the doubly charged precursor m/z 571.82. Both b-ions and y-ions confirm the core sequence of BNPsp(17-26). B) Orbitrap measurement of MS$^3$ HCD fragments of MS$^2$ product ion m/z 323.17 (b$_2$-ion carrying the modification). The detection of leucine (L) and histidine (H) immonium ions indicates that both amino acid side chains were not involved in the peptide modification. The elemental composition analysis of the unknown ion at m/z 158.11736 (a$_1$-ion) with a mass tolerance of less than 1 mmu suggests that this fragment contains the leucine residue (one nitrogen only) rather than the histidine (at least 3 nitrogens). The mass difference between the leucine immonium ion and the unknown ion is exactly the mass of the peptide’s adduct. This analysis suggests the chemical formula of C$_7$H$_{12}$N$_3$ for the adduct relative to the unmodified peptide. The comparison of C) the measured and D) the predicted $^{15}$N and $^{13}$C isotope pattern of the b$_2$-ion at m/z 323.17114 supports the calculated elemental composition.