Cardiac peptide stability, aprotinin and room temperature: importance for assessing cardiac function in clinical practice

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ABSTRACT

Brain natriuretic peptide (BNP), atrial natriuretic peptide (ANP) and N-terminal ANP are good research indices of the severity of heart failure. The stability of these peptides at room temperature has become an important factor in assessing their use as indicators of cardiac function in routine clinical practice. Inhibitors such as aprotinin are routinely added in the blood collection process, but may provide no benefit in sample collection and routine clinical practice. We assessed the stability of BNP, ANP and N-terminal ANP in blood samples collected in either the presence or the absence of the protease inhibitor aprotinin. Blood, either with or without aprotinin, was processed immediately (initial; 0 h) and after blood samples had been left for 3 h, 2 days or 3 days at room temperature. These times were chosen to reflect processing in a hospital outpatient clinic (2–3 h), or when posted from general practice (2–3 days). Initial plasma BNP, ANP and N-terminal ANP levels in the absence of aprotinin were 28.2±5.4, 44.2±7.9 and 1997±608 pg/ml respectively, and were not significantly different from initial values in the presence of aprotinin (29.0±5.9, 45.2±8.0 and 2009±579 pg/ml respectively). After 3 h at room temperature, there was a significant fall in ANP in the absence of aprotinin (36.7±7.9 pg/ml; \(P < 0.005\)), but not in the presence of aprotinin (41.2±7.6 pg/ml). Both BNP and N-terminal ANP were unchanged in either the absence (BNP, 27.6±5.5 pg/ml; N-terminal ANP, 2099±613 pg/ml) or the presence (BNP, 29.4±5.6 pg/ml; N-terminal ANP, 1988±600 pg/ml) of aprotinin. After 2 days at room temperature, ANP had fallen significantly in both the absence (16.9±3.4 pg/ml) and the presence (24.0±5.0 pg/ml) of aprotinin compared with initial values, and there was a significant difference in ANP levels in the absence and presence of aprotinin (\(P < 0.001\)). ANP levels had decreased further after 3 days at room temperature, to 11.9±3.4 pg/ml (no aprotinin) and 20.3±5.0 pg/ml (aprotinin added); these values were significantly different (\(P = 0.002\)). In contrast, there was no change in the levels of BNP or N-terminal ANP after 2 or 3 days at room temperature, in either the absence or the presence of aprotinin. These studies indicate that aprotinin adds little benefit to the stability of cardiac peptides at room temperature. Blood samples for BNP and N-terminal ANP measurement used as a test of heart function in hospital clinics and by general practitioners in the community could be taken into blood tubes containing only EDTA as anticoagulant and without the additional step of adding the routinely used inhibitor aprotinin.

INTRODUCTION

The cardiac peptides brain natriuretic peptide (BNP) and atrial natriuretic peptide (ANP) are produced predominantly within the human heart and released into the circulation [1]. Both BNP and ANP have important roles in the physiology of sodium balance and blood pressure regulation [1,2]. N-terminal ANP, a linear peptide from...
the prohormone of ANP, is also present within the circulation as a separate peptide [3]. Measurement of BNP, ANP and N-terminal proANP are now well established as useful research markers of severity and prognosis in patients with congestive heart failure [4,5] and after acute myocardial infarction [5]. In these studies [4,5], samples were processed under research conditions, with rapid freezing immediately after collection, and with the samples collected in the presence of the protease inhibitor aprotinin [4,5]. However, there is uncertainty with regard to the stability of these peptides in blood samples collected under conditions that apply in routine clinical practice [6–9]. This has important implications with regard to the use of these peptides as markers of cardiac dysfunction and prognostic outcome in patients with heart failure, particularly where blood samples may be taken in a clinical setting and dispatched for cardiac peptide (ANP, BNP, N-terminal proANP) measurement, either immediately after a hospital clinic or by first class post from a general practitioner in the community to an appropriate laboratory for peptide measurement. Also, the relative contribution of the protease inhibitor aprotinin to the stability of these cardiac peptides ANP, BNP and N-terminal ANP at room temperature has not been fully elucidated, and may, or may not, be a required step.

We investigated the stability of BNP, ANP and N-terminal proANP in human blood at room temperature and over a time scale relevant to clinical practice by measurement of their levels in aliquots of blood maintained at room temperature for up to 3 days. This delay was to allow for sufficient time for delivery of a blood sample by first class post from a general practice or regional hospital clinic, and considerably extends the time scale of our previous study from 2 [10] to 3 days. Furthermore, samples were collected in the absence and the presence of aprotinin, to assess the contribution of this routinely used inhibitor to the stability of cardiac peptides at room temperature over a 3-day period. This is of particular importance, since addition of aprotinin to blood collection tubes is an additional step commonly used in the sample collection process for cardiac natriuretic peptides [1–11]. We studied blood samples taken from heart transplant patients, since the levels of ANP, BNP and N-terminal proANP are commonly raised in such subjects [12–14].

METHODS

Patients and protocol

Ten cardiac transplant recipients were studied, all of whom attended the Harefield Transplant outpatient clinic (all male; all white), with mean age of 52.2 ± 4.1 years (range 29–68 years). All subjects gave written informed consent to the study. Peripheral venous blood samples (approx. 50 ml) were obtained from each patient. Whole blood from each patient was then placed into eight blood tubes (approx. 6 ml of blood/tube) containing EDTA as anticoagulant. Four tubes (aliquots) had aprotinin added (approx. 200 kallikrein-inhibitory units/ml of blood), and the remaining four tubes (aliquots) did not have any aprotinin added. Two tubes of blood from each patient (one containing aprotinin and one without) were centrifuged immediately after blood collection (2000 g; 10 min) to serve as baseline measurements in the presence and absence of aprotinin. The plasma from each tube was removed and stored at −80 °C. The second, third and fourth aliquots of blood (each with and without aprotinin) were allowed to stand at room temperature for 3 h, 48 h and 72 h respectively. After the appropriate time period at room temperature, each aliquot (with and without aprotinin) was centrifuged and the plasma removed and stored at −80 °C. The average room temperature was measured on each day at 10.00 hours and 17.00 hours, and was 26 °C.

Assays for plasma ANP, BNP and N-terminal proANP

Extraction procedure

For the measurement of ANP, BNP and N-terminal proANP, plasma was extracted (2.8 ml) on Sep-Pak C18 cartridges (Waters Associates) activated with 5 ml of methanol followed by 5 ml of distilled water. Plasma was drawn through the cartridge under vacuum. The cartridge was washed with 5 ml of distilled water, excess water was removed by passing air through the cartridge, and the adsorbed material was eluted from the cartridge into glass tubes containing 100 μl of BSA (RIA grade; 1 mg/ml) with 4.5 ml of acidified ethanol (4% acetic acid/80% ethanol in water). All extracts were dried down under a constant stream of air in a water bath at 50 °C. The dried extracts were reconstituted in RIA buffer (100 mmol/l sodium phosphate, 50 mmol/l NaCl, 1 g/litre BSA, 1.54 mmol/l sodium azide and 1 ml/litre Triton X-100, pH 7.3) in a 650 μl volume for each extracted sample.

RIAs for ANP, BNP and N-terminal proANP

All plasma samples taken from any individual patient were measured within the same assay for ANP, BNP and N-terminal proANP following extraction. ANP, BNP and N-terminal proANP were measured in the same reconstituted extract (100 μl in duplicate for each assay) using the RIAs developed for BNP [BNP-(1–32)] [12], ANP [ANP-(99–126)] [12] and N-terminal proANP [proANP-(1–30)] [13], as described previously. Standard curves for ANP, BNP and N-terminal proANP were constructed using synthetic human ANP-(99–126), BNP-(1–32) and proANP-(1–30) over the concentration

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Cardiac peptide stability and aprotinin ranges 3.9–500 pg/tube for ANP [12], 3.1–200 pg/tube for BNP [12] and 78.1–5000 pg/tube for N-terminal proANP-(1–30) [13]. Including the extraction procedure and subsequent measurement of ANP, BNP and N-terminal proANP by RIA, the intra- and inter-assay coefficients of variation are 11% and 12.5% respectively for ANP [12,15], 12% and 14.5% respectively for BNP [12], and 12% and 15.6% respectively for N-terminal proANP-(1–30) [13].

Statistical analysis
Data for the repeated measures of ANP, BNP and N-terminal ANP were compared by one-way analysis of variance (ANOVA). Where the ANOVA was significant, differences from basal values were tested using paired t-tests. Differences between samples at a given time point with or without aprotinin were assessed by paired t-tests. Logarithmic transformation of data was undertaken in the presence of unequal group variance as appropriate before performing the statistical tests. Due to the number of paired comparisons, a Bonferroni adjustment was applied and a P value of < 0.005 (two-tailed test) was taken as significant.

Values for plasma BNP, ANP and N-terminal proANP (pg/ml of plasma) are given as means ± S.E.M.

RESULTS

ANP, aprotinin and room temperature
Mean basal plasma ANP levels in the absence and presence of aprotinin in the 10 patients were 44.2 ± 7.9 and 45.2 ± 8.0 pg/ml respectively (Figure 1). The basal values for ANP in the presence and absence of aprotinin were not significantly different. There was a significant fall in ANP with time in both the absence (ANOVA; F = 7.6, P < 0.01) and the presence (ANOVA; F = 4.6, P < 0.025) of aprotinin. After 3 h at room temperature, there was a significant fall in ANP levels in the absence of aprotinin compared with basal values, to 36.7 ± 7.9 pg/ml (P = 0.002), but not in the presence of aprotinin (41.2 ± 7.6 pg/ml; Figure 1). After 3 h at room temperature, the mean plasma ANP level was higher in samples with aprotinin added compared with samples without aprotinin (P = 0.005; Figure 1).

After 2 days (48 h) at room temperature, the levels of ANP had fallen substantially to 16.9 ± 3.4 pg/ml (no aprotinin) and 24.0 ± 5.0 pg/ml (aprotinin added) compared with initial basal values (P < 0.001 in both cases; Figure 1). After 2 days at room temperature, plasma ANP levels were significantly higher in the samples with aprotinin compared with samples without aprotinin (P < 0.001; Figure 1).

After 3 days at room temperature, ANP levels had decreased further compared with initial basal values, to 11.9 ± 3.4 pg/ml (no aprotinin) and 20.3 ± 5.0 pg/ml (aprotinin added) (P < 0.001 in both cases; Figure 1). Plasma ANP levels were significantly higher after 3 days at room temperature in the samples with aprotinin compared with samples without aprotinin (P = 0.002; Figure 1).

BNP, aprotinin and room temperature
Mean plasma BNP levels at 0 h in the absence and presence of aprotinin in the 10 patients were 28.2 ± 5.4 and 29.0 ± 5.9 pg/ml respectively (Figure 2). The basal values for BNP in the presence and absence of aprotinin were not significantly different (Figure 2).

Figures 1–2 Stability of ANP and BNP in blood
Plasma levels of ANP measured in 10 heart transplant recipients after separation from whole blood immediately after blood collection (0 h), and after the blood sample had been left standing at room temperature (26 °C) for 3 h, 48 h or 72 h.

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Figure 2  Stability of BNP in blood
Plasma levels of BNP measured in 10 heart transplant recipients after separation from whole blood immediately after blood collection (0 h), and after the blood sample had been left standing at room temperature (26 °C) for 3 h, 48 h or 72 h. Upper panel, without aprotinin; lower panel, with aprotinin. All blood tubes contained EDTA as anticoagulant. Values (pg/ml) are plotted for each individual subject.

pared with basal values after 3 h (29.4 ± 5.6 pg/ml), 2 days (27.5 ± 5.9 pg/ml) or 3 days (28.1 ± 5.2 pg/ml) at room temperature (Figure 2). There were no significant differences in plasma BNP levels in samples without aprotinin compared with samples with aprotinin after 3 h, 2 days or 3 days at room temperature (Figure 2).

N-terminal ANP, aprotinin and room temperature
Mean plasma levels of N-terminal proANP in the absence and presence of aprotinin in the 10 patients were 1997 ± 608 and 2009 ± 579 pg/ml respectively (Figure 3). The basal values for N-terminal proANP in the presence and absence of aprotinin were not significantly different (Figure 3).

Levels of N-terminal proANP in samples collected without aprotinin were not significantly different from basal values after 3 h (2099 ± 613 pg/ml), 2 days (1985 ± 600 pg/ml) or 3 days (1816 ± 496 pg/ml) at room temperature (Figure 3). Similarly, levels in samples collected with aprotinin were not significantly different from basal values after 3 h (1988 ± 600 pg/ml), 2 days (2019 ± 596 pg/ml) or 3 days (1880 ± 477 pg/ml) at room temperature (Figure 3). There were no significant differences in plasma N-terminal proANP levels in samples without aprotinin compared with samples with aprotinin after 3 h, 2 days or 3 days at room temperature.

DISCUSSION
Our study shows that there was no difference between the basal levels of ANP in the absence and presence of aprotinin (Figure 1). However, after 3 h at room temperature there was a small difference in ANP levels (Figure 1), with 90% of ANP remaining in the presence of aprotinin, compared with 78% in its absence. These differences in ANP levels in the presence and absence of

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aprotinin were greater at 48 h and 72 h (Figure 1), with approx. 51% and 41% respectively of ANP remaining in the presence of aprotinin, but only 35% and 22% respectively of ANP remaining at 48 and 72 h in the absence of aprotinin. These findings indicate that aprotinin does have limited effects on the decrease in ANP levels in whole blood at room temperature, but only after several hours or days at room temperature (Figure 1). This is consistent with findings of a previous study for ANP and aprotinin [16], and the present study indicates that aprotinin is not a suitable inhibitor for maintaining the stability of ANP over prolonged periods of 2–3 days at room temperature (Figure 1).

In the case of BNP, there was no overall decline in plasma levels with time (Figure 2), in either the absence or the presence of aprotinin, and there were no overall differences between the levels of BNP in the absence or presence of aprotinin at any given time point. Similar results were also found in our study for N-terminal proANP, with no overall decline in the plasma levels of this peptide in either the absence or the presence of aprotinin, even when blood was kept for 2–3 days at room temperature (Figure 3). These findings clearly have important implications regarding the collection of blood samples for measurement of BNP and N-terminal ANP, since they indicate that aprotinin is not required to maintain the stability of these two peptides in human blood over 2–3 days, and could be considered an unnecessary step in the blood collection process for BNP and N-terminal ANP.

These findings are of importance, since aprotinin is used routinely in the blood collection process for measurement of cardiac natriuretic peptides, including ANP, BNP and N-terminal ANP [4,7–9,17]. Other studies have not given clear details regarding their method of blood collection [5]. One study [17] has reported stability of BNP and N-terminal ANP for up to 3 days in blood taken with aprotinin. Another study [7] has reported little effect of aprotinin on the stability of BNP and N-terminal ANP in blood at room temperature, but only measured up to a 6 h period. Our studies considerably extend this time period, and indicate that when cardiac peptides are collected in whole blood and left at room temperature for 2–3 days, aprotinin is of little value. In the case of ANP, there is an appreciable decline in peptide levels at room temperature over 2–3 days, irrespective of whether aprotinin is added to the blood collection tubes or not (Figure 1). However, aprotinin may have some benefit where sample collection, processing to plasma and freezing will take place within 2–3 h, such as in the hospital clinic setting, since aprotinin may add a small benefit over this time period (Figure 1). Assuming that ANP is to be used as a future test for cardiac dysfunction and heart failure in the community, with samples being sent by a general practitioner using first class post, then other inhibitors must be assessed.

These could include phosphoramidon, which has been shown to maintain ANP levels in whole blood over several hours [16].

In the case of BNP and N-terminal ANP, our studies clearly indicate that both peptides are stable for 2–3 days at room temperature, and therefore would be suitable to be sent through the post as whole-blood samples, with subsequent separation, freezing and measurement of these peptides at an appropriate reference laboratory.

Many studies have now shown the importance of cardiac peptides (BNP, ANP and N-terminal ANP) as indicators of cardiac dysfunction when blood samples are processed rapidly under research conditions and frozen immediately [4,5,11]. These studies have clearly shown that BNP in particular may be helpful as an indicator of outcome in patients with heart failure after acute myocardial infarction [5], and as a screening test in patients referred from primary care and suspected by their general practitioners of having heart failure [4]. In the study by Cowie et al. [4], BNP had the greatest predictive power as an indicator of heart failure when compared with ANP or N-terminal ANP. This has led to the idea of using the cardiac peptides BNP, ANP and N-terminal ANP as biochemical markers of cardiac dysfunction, in both hospitals and community practice [4,6,11], to improve the diagnosis of heart failure and to identify particular problems, such as symptomless left ventricular dysfunction [5,11]. However, the choice of which cardiac peptide to use as a biochemical indicator (ANP, BNP, or N-terminal ANP), based on the relative stability of the peptides at room temperature, has led to considerable debate [6–9]. While some authors have claimed that BNP, in particular, is stable in whole blood [6,7,10], others have found BNP to be unstable at room temperature [18]. However, this last study [18] was based on adding low-molecular-mass exogenous synthetic BNP to healthy human blood, and not on measuring endogenous BNP. Other studies [6,7,10] and our present study have investigated the stability of endogenous BNP. We have shown previously that endogenous BNP is present as both low- and high-molecular-mass forms [10]. The addition of low-molecular-mass synthetic BNP, therefore, does not necessarily reflect the conditions under which BNP circulates at raised levels in humans, as discussed previously [10]. This view is further supported by a recent study [19] demonstrating that exogenous synthetic BNP added to blood or plasma is less stable than endogenous BNP at room temperature.

Some recent studies [20–22] have also investigated the value of N-terminal proBNP as a diagnostic marker of cardiac dysfunction. However, while one study [20] demonstrated that proBNP is a good diagnostic and prognostic marker following acute myocardial infarction, other studies investigating the ability of proBNP to detect early congestive heart failure [21] or left ventricular dysfunction [22] have not found that proBNP provided
the best diagnostic information compared with N-terminal ANP or BNP. However, it has been suggested that these discrepancies could be due to the inability of the assays used in these studies [21,22] to measure accurately endogenous levels of proBNP [23]. At present, therefore, whether or not proBNP is a better diagnostic or prognostic marker than BNP requires further investigation.

Our findings that both BNP and N-terminal ANP appear stable over 2–3 days in the absence of the protease inhibitor aprotinin indicate that it is not necessary to add this inhibitor to blood collection tubes containing EDTA. This is particularly important, since it removes the need for an additional time-consuming step involving adding aprotinin to each tube before blood collection. This essentially means that standard blood tubes containing only EDTA as anticoagulant could be used for the collection of BNP and N-terminal ANP, and these are usually readily available in hospital clinics and general practice in vacutainer form. While our present study indicates good stability of BNP and N-terminal ANP in blood tubes containing EDTA as anticoagulant, the stability of both of these peptides in blood tubes containing other anticoagulants is poorly understood.

Our results showing good stability of BNP and N-terminal ANP over 2–3 days indicate that measuring plasma levels of BNP or N-terminal ANP may be useful as non-invasive tests of heart function, with samples being sent by post from a general practice or an outlying clinic to a reference laboratory for peptide measurement. This is important, given the findings that BNP and N-terminal ANP can be indicators of heart failure in the community [4], and our own findings (Figures 2 and 3) regarding the long-term stability of BNP and N-terminal ANP, even in the absence of aprotinin.

The precise reasons for the observed differences in the breakdown of cardiac peptides in whole blood are not clear, but could be due to differences in the endogenous circulating forms of ANP and BNP [10,24]. In the circulation ANP is present as a low-molecular-mass form (28 amino acids). In contrast, BNP is present as both low- (32 amino acids) and high- [probably proBNP-(1–108)] molecular-mass forms. N-terminal ANP is present in the circulation as a separate peptide from ANP, as the complete N-terminal proANP-(1–98) [3,25]. N-terminal ANP is linear, and not cyclic like ANP or BNP, and may be less susceptible to enzymic breakdown.

Clearly, our present study, using our own established methodological procedures for measurements of cardiac peptides, gives further support to investigations in which the robustness of BNP or N-terminal ANP as a test for heart failure in routine clinical practice can be assessed in more detail. These tests should now include samples from patients suspected of having heart failure being sent directly from general practice by first class post to a laboratory for measurement of BNP or N-terminal ANP levels. Since aprotinin appears to provide little additional benefit to the stability of these peptides at room temperature, such studies could now be undertaken using blood tubes containing only EDTA as anticoagulant, and without the routinely added inhibitor aprotinin.

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