Prolonged stability of brain natriuretic peptide: importance for non-invasive assessment of cardiac function in clinical practice

Martin G. BUCKLEY*, Neil J. MARCUS*, Magdi H. YACOUB* and Donald R. J. SINGER**†
*Heart Science Centre, National Heart and Lung Institute of Imperial College School of Medicine, Harefield, Middlesex UB9 6JH, U.K., and **Department of Pharmacology and Clinical Pharmacology, St George’s Hospital Medical School, Cranmer Terrace, London SW17 0RE, U.K.

Abstract

1. BNP and ANP are important research indices of severity of heart failure. However, uncertainty regarding the stability of these peptides at room temperature has limited their use to assess cardiac function in routine clinical practice.

2. We assessed the stability of BNP and ANP in blood samples left for 2 h or 2 days at room temperature compared with levels in blood processed immediately (initial). These times were chosen to reflect possible times for samples to be processed in a hospital outpatient clinic (2 h) or a blood sample posted to a laboratory from general practice (2 days). Samples were obtained from eight heart transplant recipients. Blood was separated and plasma stored immediately after collection (initial) and after 2 h or 2 days at room temperature respectively.

3. Initial plasma BNP and ANP values measured by radioimmunoassay after Sep-Pak extraction were $38.9 ± 11.1$ (S.E.M.) pg/ml and $113.6 ± 28.1$ pg/ml, respectively. After 2 h at room temperature there was no significant fall in either peptide level ($35.5 ± 9.9$ pg/ml, BNP; $104.9 ± 30.6$ pg/ml, ANP). However, after 2 days at room temperature there was a significant fall in ANP to $38.1 ± 12.6$ pg/ml ($P < 0.005$ versus initial level). In contrast, there was no significant fall in BNP after 2 days ($32.0 ± 8.4$ pg/ml). After 2 days at room temperature only $30.4 ± 4.3$ % of the ANP remained, but $86.0 ± 5.0$ % of BNP compared with the initial ANP and BNP measurements.

4. Our study clearly showed that ANP is stable for 2 h and thus could be useful as a screening test for heart disease in hospital. In contrast, BNP remained stable for 2 days. Measuring BNP may thus be practical as a test of heart function both for routine use in hospital and by general practitioners in the community.

Introduction

The cardiac peptides BNP (brain natriuretic peptide) and ANP (atrial natriuretic peptide) have important roles in the physiology of sodium balance and blood pressure regulation [1]. Measurements of BNP and ANP are now well established as useful research markers of severity and prognosis in patients with congestive heart failure.

Key words: atrial natriuretic peptide, brain natriuretic peptide, cardiac function, stability, room temperature, molecular forms.

Abbreviations: ANOVA, analysis of variance; ANP, atrial natriuretic peptide; BNP, brain natriuretic peptide.

Correspondence: Dr M. G. Buckley, Heart Science Centre, National Heart and Lung Institute of Imperial College School of Medicine, Harefield UB9 6LH, Middlesex, U.K.
[2,3] and after acute myocardial infarction [3] when samples are processed under research conditions with rapid freezing immediately after collection. However, there is uncertainty regarding the stability of these peptides in blood samples collected under conditions that apply in routine clinical practice [4–7]. This has important implications regarding 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 BNP and ANP measurement either immediately after a hospital clinic, or by first class post by a general practitioner in the community to an appropriate laboratory for peptide measurement. We investigated the stability of BNP and ANP in human blood at room temperature and over the timescale relevant to clinical practice by measurements in aliquots of blood maintained at room temperature for up to 2 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. We studied blood samples from heart transplant patients as they typically have raised levels of both BNP and ANP [8]. We also studied whether high-molecular-mass forms of BNP circulate as one possible explanation for any differences in stability of ANP compared with BNP.

**METHODS**

We studied eight cardiac transplant recipients attending the Harefield Transplant outpatient clinic for elective review (one female and seven males; five were Caucasian and three Asian) with a mean age of 54.5 ± 3.3 (range 39–71) years. All subjects gave written informed consent to the study, which was approved by the local hospital ethics committee. Peripheral venous blood samples were obtained from each patient and collected as previously described [8] into tubes containing EDTA and Trasylol (aprotinin) and split into three aliquots. One aliquot of blood from each patient was centrifuged immediately after collection and the plasma frozen at −80 °C. The second and third aliquots of blood were allowed to stand at room temperature for 2 h and 48 h respectively, before centrifugation, plasma removal and storage at −80 °C. Average room temperature measured at 10:00 and 17:00 h on each day was 22 °C.

**Assay for plasma BNP and ANP**

All plasma samples were measured within 1 week of collection and within the same assay for BNP and ANP respectively, as previously described [8]. Both BNP and ANP were extracted from 2.5 ml of plasma on Sep-Pak C-18 cartridges as previously described [8], and reconstituted into a final volume (0.5 ml) of radioimmunoassay (RIA) buffer [8]. Both BNP and ANP were measured in the same reconstituted extract using the RIA for BNP (BNP 1-32) and ANP (ANP 99-126) as previously described [8]. Standard curves for BNP and ANP were constructed using synthetic BNP (1-32) and ANP (99-126) over the concentration range of 3.1–200 pg/tube for BNP and 3.9–500 pg/tube for ANP [8]. The limit of sensitivity of the BNP and ANP RIA was 3.1 and 3.9 pg/tube. Including the extraction procedure and subsequent measurement of BNP and ANP by RIA, the intra- and inter-assay coefficients of variation for BNP are 12% and 14.5% respectively, and 11% and 12.5% for ANP [8,9]. There is no cross-reactivity for ANP in the BNP RIA, and no cross-reactivity for BNP in the ANP RIA when both are tested over the range 3.1–20000 pg/tube [8].

**Gel filtration of extracted plasma**

Gel filtration of extracted plasma for investigation of molecular circulating forms of BNP and ANP was undertaken using methods similar to those described previously [10]. The gel filtration column was calibrated using synthetic human BNP (1-32) and ANP (99-126) standards (25 ng). Void volume (V₀) was estimated with Dextran Blue (2 mg/ml; molecular mass 2 × 10⁶ Da). Pooled plasma taken from cardiac transplant recipients (48 ml) was extracted on Sep-Pak cartridges, reconstituted in a 0.5-ml volume of assay buffer [10] and loaded on to the column. Fractions collected were tested for immunoreactivity using the above BNP and ANP RIAs [8].

Results for plasma BNP and ANP are expressed as means ± S.E.M. Data for the repeated measurements of plasma BNP and ANP were compared by one-way analysis of variance (ANOVA) for repeated measures. For significant differences in the ANOVA, paired data were then compared by t-tests using the variance from the ANOVA. A P value of less than 0.05 for a two-tailed test was taken to be significant.

**RESULTS**

**Plasma BNP and ANP**

Immediately after collection, mean plasma BNP and ANP levels in the eight patients were 38.9 ± 11.1 and 113.6 ± 28.1 pg/ml respectively (Figure 1). After 2 h at normal room temperature there was no significant fall in either BNP or ANP levels with values of 35.5 ± 9.9 and 104.9 ± 30.6 pg/ml (Figure 1). However, after 48 h at room temperature there was a significant fall in the plasma level of ANP to 38.1 ± 12.6 pg/ml compared with the initial ANP value (P < 0.005, Figure 1). In
Stability of brain and atrial natriuretic peptide

Figure 1  Plasma level of BNP and ANP measured in eight heart transplant recipients after separation from whole blood immediately after blood collection (0 h), and after 2 h and 48 h of blood left standing at room temperature (22 °C). Values are plotted for each individual subject.

In contrast, there was no significant fall in the plasma level of BNP with a mean value of 32.0 ± 8.4 pg/ml after 48 h at room temperature (Figure 1). The level of ANP after 48 h at room temperature was only 30.4 ± 4.3% of the initial ANP value. In contrast, after 48 h at room temperature, BNP levels remained at 86.0 ± 5.0% of initial values.

Gel filtration of extracted plasma

After gel filtration of extracted plasma from cardiac transplant recipients, BNP immunoreactivity occurred as two separate and distinct peaks (Figure 2). A low-molecular-mass peak eluted at 33–35 ml which corresponded to the elution volume for the synthetic human BNP (1–32) standard, and a high-molecular-mass peak eluted at 18–20 ml which corresponded to the void volume (Vo). Measurement of ANP in the same fractions revealed a single peak of immunoreactivity at 38–40 ml which corresponded to the elution volume of the synthetic human ANP (99-126) standard (Figure 2).

DISCUSSION

Our study shows that BNP and ANP levels were little changed over 2 h at room temperature in whole blood. ANP levels then decreased by two-thirds in samples kept at room temperature for 2 days. In contrast, almost 90% of the BNP activity was still present in whole blood after 2 days at room temperature. Thus both BNP and ANP appear sufficiently stable at room temperature for practical use in the hospital clinic setting as indices of cardiac function. Our results in samples studied for up to 2 days greatly extend those of some previous studies [5] which reported stability of BNP in plasma samples tested up to 6 h at room temperature. Our results in samples taken from cardiac transplant recipients are also consistent with a recent study in patients with heart disease [11] which also reported stability of BNP for a slightly longer period of up to 3 days in whole blood at room temperature, supporting our own findings in a different patient group.

Tsuji et al. [12] noted that BNP levels reduced in activity at room temperature to 60% at 3 h and to 10% by 24 h. However, those results were based on measurements in plasma from healthy subjects to which synthetic BNP had been added [12]. In contrast, studies by us and others reporting stability of BNP have been based on assays measuring high levels of endogenous BNP in
One study [13] has previously reported that plasma ANP is not stable under any storage conditions, even when stored at sub-zero temperatures. In contrast, we have found that high levels of plasma ANP taken from patients with heart failure are stable for at least 6 months when stored at sub-zero temperatures [9]. More recently, studies have also demonstrated 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 [2,3]. 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 [3], and as a screening test in patients suspected by their general practitioners of having heart failure [2]. This has led to the idea of using the cardiac peptides BNP, ANP and N-terminal proANP as biochemical markers of cardiac dysfunction both in hospitals and in community practice [2,4], to improve diagnosis of heart failure and identify particular problems such as symptomless left ventricular dysfunction [3]. However, the choice of which cardiac peptide to use as a biochemical indicator (ANP, BNP, N-terminal ANP), based on relative stability of the peptides at room temperature, has led to considerable confusion and debate [4–7]. While some authors have claimed that BNP, in particular, is stable in whole blood [4,5], others have found BNP to be unstable at room temperature [12]. One recent study [2] has reported the value of BNP as a screening test for heart failure in patients referred from primary care. In this study [2], BNP was shown to have a greater predictive power as an indicator of heart failure in patients from the community than either ANP or N-terminal ANP. However the measurements were made in samples handled under very rigorous research conditions, with blood samples collected from patients being processed immediately, and plasma stored at sub-zero temperatures [2].

Our results showing stability of BNP over 2 days indicate that measuring plasma BNP may also be useful as a simple non-invasive test of heart function, with samples sent by post from a general practice or an outlying clinic to a reference laboratory for BNP measurement. This would have major advantages over current imaging methods in general clinical use for assessing cardiac function. The ability of clinicians in hospital and in general practice to use BNP as a more simple practical clinical test of cardiac function could reduce costs, inconvenience and delays involved in more complex methods of assessment. This is important, given the finding that BNP can be an indicator of heart failure in the community [2], and our own finding (Figure 1) regarding long-term stability of BNP.

However, the relative contribution of aprotinin (a protease inhibitor) to the stability of cardiac peptides (BNP, ANP and N-terminal ANP) at room temperature requires further investigation, since most studies have involved the addition of aprotinin to blood tubes before sample collection at room temperature and subsequent storage at sub-zero temperatures [2,5,6,7,11]. The presence of aprotinin has been reported to have little effect on BNP and N-terminal ANP stability in blood at room temperature, but only measured up to a 6-h period [5]. Other studies have not given clear details regarding their method of blood collection [3]. In our study, ANP levels declined substantially after 2 days at room temperature, even in the presence of aprotinin, and this is consistent with a previous finding for ANP [14], although BNP was not investigated in that study. The possible use of other protease inhibitors to stabilize ANP at room temperature for sample collection could also be tested further, since one study has reported greater stability of ANP at room temperature in the presence of phosphoramidon [14], although BNP and N-terminal ANP were not measured. Clearly, the addition of protease inhibitors to blood collection tubes used for cardiac peptide sampling is an additional step, commonly used in the blood collection process. If cardiac peptides are to be used as diagnostic and prognostic indicators in hospital clinics and community practice, further work is required to examine whether or not protease inhibitors should be added to blood collection tubes, and to determine which is the most appropriate inhibitor. If BNP in particular is as stable in the absence of aprotinin, it would not be necessary to add this when collecting samples in hospital clinics and routine clinical practice. Clearly, our study gives support to further investigations in which the robustness of BNP as a test for heart failure in routine practice is assessed in more detail. These should include samples from patients suspected of having heart failure being sent direct from the general practice by first class post to the laboratory for BNP measurement.

ACKNOWLEDGMENTS

This study was supported in part by Harefield Hospital Research Trust. M.G.B. is an Imperial College School of Medicine Non-Clinical Lecturer.
REFERENCES

11 Murdoch, D. R., Byrne, J., Morton, J. J. et al. (1997) Brain natriuretic peptide is stable in whole blood and can be measured using a simple rapid assay: implications for clinical practice. Heart 78, 594–597

© 1998 The Biochemical Society and the Medical Research Society