Assessment of the stability of N-terminal pro-brain natriuretic peptide *in vitro*: implications for assessment of left ventricular dysfunction

P. F. DOWNIE, S. TALWAR, I. B. SQUIRE, J. E. DAVIES, D. B. BARNETT and L. L. NG
Department of Medicine and Therapeutics, University of Leicester, Robert Kilpatrick Clinical Sciences Building, Leicester Royal Infirmary, Leicester LE2 7LX, U.K.

A B S T R A C T

Plasma concentrations of N-terminal pro-brain natriuretic peptide (NT-proBNP) are raised in patients with left ventricular dysfunction. Measurement of this peptide has a potential diagnostic role in the identification and assessment of patients with heart failure. The stability of this peptide over time periods and conditions pertaining to routine clinical practice has not been reported previously. Blood samples were obtained from 15 subjects. One aliquot was processed immediately, and the remaining portions of the blood samples were stored for 24 h or 48 h at room temperature or on ice prior to processing. Plasma concentrations of NT-proBNP were measured with a novel immunoluminometric assay developed within our laboratory. Mean plasma concentrations of NT-proBNP were not significantly different whether blood samples were centrifuged immediately and stored at $-70\,^{\circ}C$ or kept at room temperature or on ice for 24 h or 48 h. The mean percentage differences from baseline (reference standard) were $+5.2\%$ (95% confidence interval $+18.2$ to -7.8%) and $+0.8\%$ ($+15.2$ to -13.7%) after storage for 24 h at room temperature or on ice respectively, and $+8.9\%$ ($+24.2$ to -6.5%) and $+3.2\%$ ($+15.1$ to -0.9%) for storage for 48 h at room temperature or on ice respectively. Pearson correlation coefficients for baseline NT-proBNP concentrations compared with levels at 48 h at room temperature or on ice were $r = 0.89$ and $r = 0.83$ respectively (both $P < 0.0001$). Thus NT-proBNP extracted from plasma samples treated with EDTA and aprotinin is stable under conditions relevant to clinical practice.

INTRODUCTION

Heart failure, defined as symptomatic left ventricular systolic dysfunction (LVSD) [1,2], affects up to 500 000 people in the U.K. [3–5]. The incidence of this condition is expected to rise substantially over the next 10 years [6]. Furthermore, the prevalence of asymptomatic LVSD rivals that of symptomatic LVSD [1]. Heart failure mortality data are comparable with those for severe malignant disease. Five-year survival rates are 25% in men and 38% in women [7].

The adequacy of clinical diagnosis of heart failure is poor, with reported levels of false-positive diagnoses ranging from 30 to 50% [8]. Improved diagnosis of symptomatic or asymptomatic LVSD would allow for earlier intervention of known therapeutic benefit, namely use of angiotensin-converting enzyme inhibitors [9,10].

Plasma levels of N-terminal pro-brain natriuretic peptide (NT-proBNP) are elevated in stage 1 of heart disease (NYHA criteria) [11], and the proportional and absolute levels of NT-proBNP exceed those of BNP-32 (BNP containing 32 amino acids in the peptide chain) in

patients with cardiac impairment [11]. NT-proBNP is a potentially more discerning marker of cardiac impairment than BNP-32. Interest has therefore centred on the potential role that natriuretic peptides may play as aids to the diagnosis of LVSD. However, central to the widespread applicability of a biochemical test in determining the degree of functional cardiac impairment is the stability of the marker itself. In view of this, we set out to assess the stability of NT-proBNP over a set of various time scales and conditions.

METHODS

Materials
The methyl acridinium ester [4-(2-succinimidyloxy-carbonylethyl)phenyl-10-methylacridinium 9-carboxylate fluorosulphonate] was a gift from Dr. Stuart Woodhead and Dr. Ian Weeks (Molecular Light Technology Ltd., Cardiff, Wales, U.K.). Paramagnetic particles coated with goat anti-(rabbit IgG) were from Metachem Diagnostics Ltd. (Northampton, U.K.). The C_{18} plasma extraction columns were obtained from Peninsula Laboratories. Assays were performed using a Berthold Autolumat LB953 luminometer.

Subjects
A total of 15 subjects (nine male/six female; median age 62 years; range 21–84 years) were enrolled for involvement in the study. Of these, seven subjects had a confirmed diagnosis of an acute myocardial infarction, three subjects had a diagnosis of unstable angina, and five others had no cardiovascular history. All subjects gave informed consent for the study, which was approved by the local ethical research committee.

Collection and storage of blood samples
Patients remained in a relaxed supine position for 20 min prior to blood sampling. A sample of 25 ml of peripheral venous blood was drawn from an antecubital fossa vein of each subject and collected into pre-chilled polypropylene tubes containing EDTA (1 mg/ml of blood) and aprotinin (500 kallikrein-inhibitory units/ml of blood), and placed immediately on ice.

Each blood sample was divided into five 5 ml aliquots. One aliquot was centrifuged (2700 g for 10 min at 4 °C) and the plasma was stored at −70 °C within 30 min of collection. Other blood samples were stored for 24 or 48 h at room temperature or on ice, and then separated and stored at −70 °C. The average room temperature measured at 10.00 and 17.00 hours on each day was 24 °C.

Plasma extraction was required prior to assay. C_{18} plasma extraction columns were primed with 1 ml of 60 % (v/v) acetonitrile/1 % (v/v) trifluoroacetic acid (TFA) in water, followed by 9 ml of 1 % (v/v) TFA.

Plasma samples were thawed, acidified with an equal volume of 1 % (v/v) TFA and loaded on to the cartridges. Columns were washed with 6 ml of 1 % (v/v) TFA and eluted with 2 ml of 60 % (v/v) acetonitrile/1 % (v/v) TFA in water; the solvent was then evaporated to dryness under vacuum. Samples were stored at −70 °C until assay.

Immunoluminometric assay for NT-proBNP
All samples were assayed within 4 weeks of collection, and all samples from each patient were processed simultaneously. NT-proBNP was measured with a novel immunoluminometric assay previously developed within our laboratory [12]. We used an in-house rabbit anti-(human NT-proBNP) polyclonal antibody directed against a domain in the C-terminal section of NT-proBNP (amino acids 65–76). The peptide was labelled using the chemiluminescent label 4-(2-succinimidyloxy-carbonylethyl)phenyl-10-methylacridinium 9-carboxylate fluorosulphonate [12]. All samples were assayed in duplicate, and the average of the two measurements is reported. The assay has been demonstrated to be specific for NT-proBNP, and unreactive with atrial natriuretic peptide, BNP or C-type natriuretic peptide [12]. The within-assay and between-assay coefficients of variation were 3.0 % and 11.2 % respectively (at 30 fmol/tube). Coefficients of variation between and within assays as previously reported are acceptable for a competitive assay [12].

Statistical analysis
All analyses of NT-proBNP concentrations were carried out after logarithmic transformation, as the data were not normally distributed. Differences among the various approaches for handling the samples before storage at −70 °C were assessed by analysis of variance and the paired t test, and corresponding 95 % confidence intervals are reported. These confidence intervals were transformed into percentage differences to aid interpretation. All statistical analyses were carried out using the software package Minitab (Minitab Inc.). Values with $P < 0.05$ were considered significantly different.

RESULTS

Plasma NT-proBNP concentrations ranged from 90 to 557 fmol/ml (mean 226.8 fmol/ml) at baseline (normal range < 200 fmol/ml [12]).

There were no significant differences in mean plasma concentrations of NT-proBNP between samples that were processed immediately and those that were kept at room temperature or on ice for 24 h or 48 h (Table 1).
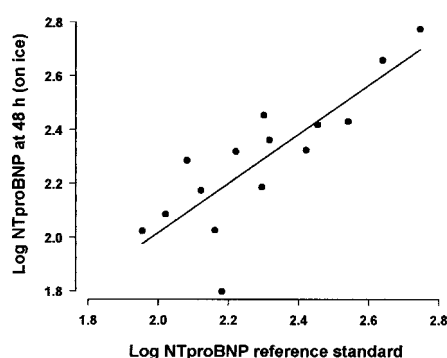
Pearson correlation coefficients for baseline NT-proBNP levels (reference standard) compared with levels at 24 h in samples kept at room temperature or on ice

Table I   Plasma concentrations of NT-proBNP after storage under different conditions

Storage conditions	Difference from reference standard		95% Confidence interval	
	(fmol/ml)	(%)	(fmol/ml)	(% of reference standard mean)
24 h at room temperature	11.8	5.2	+41.4 to −17.8	+18.2 to −7.8
24 h on ice	1.7	0.8	+34.6 to −31.2	+15.2 to −13.7
48 h at room temperature	20.1	8.9	+55.1 to −14.8	+24.2 to −6.5
48 h on ice	7.5	3.2	+35.1 to −20.0	+15.1 to −0.9
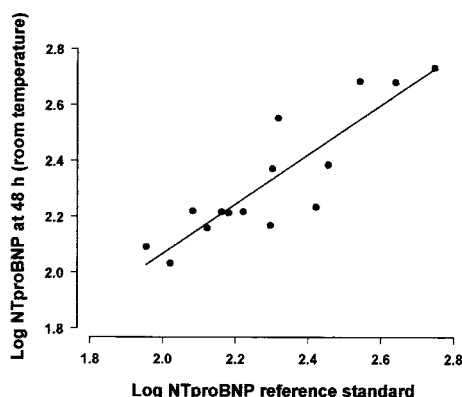
Figure 1   Correlation between NT-proBNP concentrations in plasma stored immediately at −70 °C and in that kept for 48 h as whole blood at room temperature

The data have been logarithmically transformed.

Figure 2   Correlation between NT-proBNP concentrations in plasma stored immediately at −70 °C and in that kept for 48 h as whole blood on ice

The data have been logarithmically transformed.

were $r = 0.86$ and $r = 0.78$ respectively ($P < 0.0001$ and $P = 0.001$ respectively). Pearson correlation coefficients for baseline NT-proBNP levels compared with levels at 48 h in samples kept at room temperature or on ice were $r = 0.89$ and $r = 0.83$ respectively (both $P < 0.0001$) (Figures 1 and 2).

DISCUSSION

To our knowledge, this is the first study assessing the stability of NT-proBNP under conditions pertaining to those applicable in routine clinical practice. Our results demonstrate that NT-proBNP is stable in whole blood treated with EDTA and aprotinin for up to 48 h both at room temperature and when stored on ice. We therefore conclude that the stability of NT-proBNP confers the potential for its introduction into wider clinical practice as an additional tool in the assessment of left ventricular systolic function. The relevance of this conclusion is heightened because of the potential superiority NT-proBNP has over BNP-32 as a marker of LVSD [11]

The potential applications of the assay of NT-proBNP are widespread. Circulating levels of NT-proBNP measured 2–4 days after acute myocardial infarct have already been shown to predict left ventricular function and 2-year survival [13]. Thus routine assay of NT-proBNP may assist in the risk stratification of the post-infarct population, a group clearly at high risk of progressing to symptomatic heart failure. However, the prolonged stability of NT-proBNP will also facilitate its use by community-based general practitioners for diagnostic assessment of patients with suspected LVSD, when access to investigations such as echocardiography or radionuclide ventriculography are limited by expense, inconvenience or availability of equipment or expertise.

The use of a biochemical marker will clearly not completely replace established methods of assessing ventricular dysfunction, such as echocardiography and radionuclide ventriculography. However, circulating levels of NT-proBNP may identify those patients (particularly when aimed at high-risk individuals) in whom further investigation is required, and is an additional aid to clinical assessment alone. This would limit inappropriate and unnecessary investigations, and moreover would allow for the earlier identification of patients with either symptomatic and asymptomatic LVSD. Such a strategy would facilitate the introduction of therapies of proven therapeutic benefit [9,10], such as angiotensin-converting enzyme inhibitors, at a time when the potential for clinical benefit is at a maximum. Equally,

normal levels of NT-proBNP may identify those patients with preserved left ventricular function in whom cardiovascular investigations are not indicated.

The results of this work are encouraging. The potential use of the assay of NT-proBNP as an aid to the identification of patients with LVSD, especially in general-practice-based screening programmes, requires further and more detailed investigation.

ACKNOWLEDGMENTS

REFERENCES

1 McDonagh, T. A., Morrison, C. E., Lawrence, A. et al. (1997) Symptomatic and asymptomatic left-ventricular systolic dysfunction in an urban population. Lancet 350, 829–833

2 Task Force on Heart Failure of the European Society of Cardiology (1995) Guidelines for the diagnosis of heart failure. Eur. Heart. J. 16, 741–751

3 Sutton, G. C. (1990) Epidemiological aspects of heart failure. Am. Heart. J. 120, 1538–1540

4 Smith, W. M. (1985) Epidemiology of congestive heart failure. Am. J. Cardiol. 55, 3A–8A

5 McMurray, J., McDonagh, T., Morrison, C. E. and Dargie, H. J. (1993) Trends in hospitalization for heart failure in Scotland 1980–1990. Eur. Heart J. 14, 1158–1162

6 Bonneux, L., Barendregt, J. J., Meeter, K., Bonsel, G. J. and van der Mass, P. J. (1994) Estimating clinical morbidity due to ischaemic heart disease and congestive heart failure: the future rise of heart failure. Am. J. Public Health 84, 20–28

7 Ho, K. K., Anderson, K. M., Kannel, W. B., Grossman, W. and Levy, D. (1993) Survival after the onset of congestive heart failure in Framingam Heart Study subjects. Circulation 88, 107–115

8 Remes, J., Miettinen, H., Reunan, A. and Pyorala, K. (1991) Validity of clinical diagnosis of heart failure in primary health care. Eur. Heart J. 12, 315–321

9 The CONSENSUS Trial Study Group (1987) Effects of enalapril on mortality in severe congestive heart failure: results of the Cooperative North Scandanavian Enalapril Survival Study (CONSENSUS). N. Engl. J. Med. 316, 1429–1435

10 SOLVD Investigators (1992) Effect of enalapril on mortality and the development of heart failure in asymptomatic patients with reduced left ventricular ejection fractions. N. Engl. J. Med. 327, 685–691

11 Hunt, P. J., Richards, A. M., Nichos, M. G., Yandle, T. G., Doughty, R. N. and Espiner, E. A. (1997) Immunoreactive amino-terminal pro-brain natriuretic peptide (NT-proBNP): a new marker of cardiac impairment. Clin. Endocrinol. 47, 287–296

12 Hughes, D., Talwar, S., Squire, I. B., Davies, J. E. and Ng, L. L. (1999) An immunoluminometric assay for N-terminal pro-brain natriuretic peptide: development of a test for left ventricular dysfunction. Clin Sci. 96, 373–380

13 Richards, M. A., Nicholls, M. G., Yandle, T. G. et al. (1998) Plasma N-terminal pro-brain natriuretic peptide and adrenomedullin: new neurohormonal predictors of left ventricular function and prognosis after myocardial infarction. Circulation 97, 1921–1929