The effect of class-specific protease inhibitors on the stabilization of B-type natriuretic peptide in human plasma

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Abstract

Background: B-type natriuretic peptide (BNP) is a cardiac hormone that regulates hemodynamic equilibrium. In the circulation, its activity is controlled by proteolytic factors. Accurate measurement of BNP in a patient’s plasma may be affected by degradation due to proteolysis. Objective: We report on the identification and performance of classes of protease inhibitors that stabilize BNP in plasma. Design and methods: Using the Bayer ADVIA Centaur® BNP assay, we measured the effect of arginine, serine and/or specific kallikrein protease inhibitors (PIs) on exogenous spiked or endogenous BNP in patient plasma. Results: Compared to controls without inhibitor, all PIs were capable, to varying degrees, of retarding the rate of proteolytic degradation. The kallikrein-specific inhibitor, D-Phe–Phe–Arg–chloromethylketone (PPACK II) was most effective as a single constituent and was able to eliminate BNP degradation in patient samples for up to 6–10 days when stored at 2–8 °C. Conclusions: The stability of BNP was markedly increased in the presence of kallikrein-specific PPACK II and a broad spectrum of serine PIs. Use of these compounds offers a simple method of extending sample handling and storage of plasma samples containing BNP.

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1. Introduction

B-type natriuretic peptide or brain natriuretic peptide (BNP) is a specific and sensitive indicator of heart failure (HF). BNP is a vasoactive cardiac peptide hormone that is synthesized and secreted into the bloodstream primarily from the heart ventricles [1,2]. Its mature biologically active form is composed of 32 amino acids derived from the C terminus (amino acids 77–108) of 108-amino-acid-long proBNP molecule. The hormone promotes natriuresis and diuresis, acts as a vasodilator and antagonizes the vasoconstrictor effects of the renin–angiotensin–aldosterone system [1–3].

Because plasma concentrations of BNP increase with the decline of heart function, particularly ventricular efficiency, the measurement of the BNP
concentration in blood is useful for the diagnosis and prognosis of HF and acute myocardial infarction (AMI) [4–6]. Since heart disease and HF are major health problems worldwide, BNP has been proposed as a biochemical marker for use in screening patients to select for further cardiac investigations and/or treatment [3]. Application of BNP as a diagnostic marker for identifying patients with heart disease, e.g., left-ventricular systolic and/or diastolic dysfunction, is complicated by the peptide’s limited stability in blood samples [7–11]. Natural clearance of natriuretic peptides out of circulation is controlled by specific receptor-mediated mechanisms and proteolysis by neutral endopeptidases (NEP). NEPs are cell membrane-associated ectoenzymes that carry out vasopeptidase activity [12,13]. In vitro, endogenous BNP and synthetic forms of the peptide are prone to relatively rapid decomposition in blood plasma and serum as a result of proteolysis. For example, degradation of BNP occurs within 24 h of separation of plasma from whole blood. This degradation progresses even on storage under refrigerated conditions, making it difficult to accurately measure BNP in plasma samples after 24 h, unless the sample is then frozen. In general, separated plasma retains greater than 90% of its initial BNP concentration after 24 h at 2–8 °C that degrades up to 60% or more by 72 h [9,10,14,15]. Unseparated whole blood imparts additional stability to BNP, as plasma prepared from whole blood after 24-h storage unrefrigerated retains 96% of its initial content [10,14,15].

Although approaches for stabilizing BNP in blood samples have been reported in the literature, none is completely satisfactory, and most are inefficient. Currently, BNP stability in samples is mostly maintained using precautions during blood collection and pretest storage. Attempts to stabilize BNP in patient blood samples have involved the use of plastic collection tubes [7], the addition of EDTA to blood samples [10] and the addition of a combination of aprotinin and benzamidine to blood samples [11].

Eliminating or significantly reducing BNP degradation post-phlebotomy is an important goal. A simple method for stabilizing BNP after sample collection would allow laboratorians extra time for repeat testing or additional analysis prior to selection and freezing of samples that need to be retained. The present study identifies several classes of protease inhibitors (PIs) that meet the above objectives.

2. Methods

2.1. Materials

Synthetic BNP (amino acids 77–108) was acquired from Phoenix Pharmaceuticals (Belmont, CA, USA); normal human EDTA–plasma and human serum albumin (HSA) were obtained from Serologicals (Norcross, GA, USA). Data used in elucidating epitope specificity of the BNP antibodies used in both the ADVIA Centaur and Biosite® Triage® assays was obtained through the courtesy of Dr. H. Shimizu (Shionogi & Co., Osaka, Japan). A general epitope map was constructed using synthetic BNP fragments truncated to various degrees from the C and N terminus. In this study, the nomenclature, BNP and BNP-32, refer to the biologically active peptide. PIs were purchased from Calbiochem-Novabiochem (San Diego, CA, USA). Tested inhibitors, their chemical compositions, and abbreviations or conventional names are AEBSF ([4-(2-aminoethyl)benzenesulfonylfluoride]), antipain (N-(Nα-carbonyl-Arg–Val–Arg)Phe), benzamidine (benzenecarboximidamide), aprotinin, DFP (diisopropylfluorophosphate), leupeptin (acetyl-Leu–Leu–Arg), PPACK I (D-Phe–Pro–Arg–chloromethylketone) and H-D-Phe–Phe–Arg–chloromethylketone (PPACK II). Studies on endogenous BNP stabilization were performed on human samples with elevated levels of BNP.

Samples were obtained in accordance with criteria specified in the United States Code of Regulations, Federal Policy for the Protection of Human Subjects (45GFR 46.1). Sample specimens were obtained from the following centers: Hartford Hospital (Hartford, CT), Promeddx (Norton, MA, USA) and Quest Laboratories (San Juan Capistrano, CA, USA).

2.2. Selection of protease inhibitors

In separate experiments, we investigated the effects of various PIs either singly or in combination to prevent degradation of exogenous synthetic BNP spiked into pooled human EDTA plasma. Exogenous BNP concentrations ranged from 300 to 1000 pg/ml. At various time intervals, sample aliquots with or
without the addition of PIs were transferred to plastic containers and immediately frozen to prevent further degradation. Final PI concentrations in plasma after addition of inhibitor from its concentrated solution to the blood sample ranged from <1 to 100 μg/ml plasma for competitive inhibitors antipain, leupeptin, aprotinin and PPACK I and II. For AEBSF, benzamidine and DFP only single high concentrations were tested. PI concentrations reflect total inhibitor mass without correction for purity (~70–90%). Observed rapid BNP degradation in EDTA plasma suggested that proteolysis is not directly related to NEP. NEP inhibitors (Zn chelators and phosphoramidon) were tested previously and found to be ineffective (data not shown).

2.3. BNP testing

BNP concentrations were measured using the ADVIA Centaur® immunoassay analyzer (Bayer Diagnostics, Tarrytown, NY). This method employs two BNP-specific monoclonal antibodies recognizing two distinct epitopes of the peptide. The capture antibody, recognizes the C terminus of BNP (amino acids 27–32) and is biotinylated. The detector antibody is labeled with acridinium ester and recognizes the ring structure of the molecule (amino acids 14–21). Both antibodies are documented in the clinical literature and have been used in commercial BNP assays since 1993 (Shionoria™, Shionogi & Co.) [2,5,14–17]. Immunocomplexes formed during incubation of BNP-containing samples with labeled antibodies were separated using streptavidin-coated paramagnetic particles and quantified chemiluminometrically.

2.4. Stabilization effect of PPACK II solutions on BNP in plasma

To develop a simple to use stabilizing agent for BNP in plasma, concentrated stocks of PPACK II at 3500 μg/ml in distilled water were prepared such that the addition of 1 drop (~35 μl) would contain sufficient inhibitor to protect against proteolytic degradation in <1–4 ml plasma. These volumes are sufficient to account for the maximum plasma yield of typical blood collection tubes. Bottles of reconstituted PPACK II kept at −20 °C were thawed then stored at 4 °C for up to 2 weeks to test for their ability to prevent degradation of exogenous BNP in 1, 2, or 4 ml of plasma. BNP stability in plasma after addition of PPACK II was tested for up to 2 weeks. At each time interval, an aliquot of plasma (with inhibitor added) was removed from storage at 4 °C and immediately frozen in order to prevent further degradation. At the conclusion of the incubation period, all aliquots were thawed and tested simultaneously on the ADVIA Centaur BNP assay. The BNP recovery was compared to the original aliquot frozen at time 0 (control material).

2.5. Stability of PPACK II stock solutions

Additional experiments were performed to assess the stability of reconstituted PPACK II inhibitor after multiple freeze–thaws and storage under refrigerated conditions. To test the stability of the PPACK II solution after immediately being thawed and after 1 week storage at 4 °C, aliquots of exogenous BNP (130 pg/ml) mixed in human plasma were either spiked immediately with PPACK II and stored at 4 °C or frozen without PPACK II to prevent degradation. Freshly spiked plasma aliquots had PPACK II added immediately. Frozen aliquots of BNP plasma were thawed after 1 week, followed by addition of 1-week-old PPACK II stored at 4 °C. These plasma aliquots were then stored up to an additional 2 weeks at 4 °C. PPACK II bottles also underwent up to three freeze–thaw cycles to assess their ability to inhibit BNP degradation for an additional 2 weeks when added to plasma-containing BNP. Following PPACK II addition, all BNP samples were tested after 0, 1 and 2 weeks storage at 4 °C.

2.6. Stabilization of endogenous BNP in patient plasma samples

Individual patient samples with BNP concentrations ranging from 12 to >2800 pg/ml with and without protease inhibitor were tested for stability from periods of 3 h to 6 days. Studies without PI addition evaluated the effects of room temperature and refrigerated storage on degradation of endogenous BNP in patient plasma samples.
3. Results

Common classes of serine protease inhibitors tested included competitive inhibitors, antipain and leupeptin, irreversible inhibitors, AEBSF and DFP, and kallikrein-specific PPACK II. These were compared for their ability to inhibit BNP degradation to both aprotinin and benzamidine, the molecules that have been previously reported to stabilize BNP in human plasma [11]. Competitive inhibitors and PPACK II were titrated at various concentrations, while irreversible inhibitors were tested at a single high dose. Since EDTA, an effective inhibitor of vasopeptidase activity, was used at ~5 mM as anticoagulant, it was presumed that related metalloproteases were not the source of proteolytic degradation of BNP in these plasma samples.

3.1. Peptidyl arginine aldehyde protease inhibitors, antipain and leupeptin

Arginyl residues are often present in sites targeted by proteolysis. The BNP amino acid sequence contains four arginine residues at amino acid residues 13, 17, 30 and 31 (Fig. 1). The peptidyl arginine aldehyde PIs, antipain and leupeptin, demonstrated similar effects in reducing BNP degradation in plasma (Fig. 2A and B). Without inhibitor, recovery of exogenous synthetic BNP spiked into plasma at ~800–900 pg/ml dropped to ~10% of its initial concentration after 40 h of storage at 4 °C and was undetectable by the immunnoassay after 144 h of storage at 4 °C. In the presence of 50 µg/ml of antipain or leupeptin, recovery of BNP after 6 days storage at 4 °C was unchanged compared to the initial measurements at the time of preparation (828.3 pg/ml vs. 822.2 pg/ml antipain; 792 pg/ml vs. 835.9 pg/ml leupeptin).

3.2. Kallikrein-specific inhibitors

Fig. 3 shows the effect of specific kallikrein inhibitors on BNP stability in plasma. This type of inhibitor was selected due to the role of kallikreins in kinin generation in the renin–angiotensin–aldosterone system, blood coagulation pathways and other systems requiring conversion of zymogens to active enzymes [18]. The association between contact activation of the factor XII to XIIa followed by conversion of prekallikrein to kallikrein in the extrinsic pathway of blood coagulation has been implicated as the cause of increased rates of BNP degradation in glass vs. plastic tubes and in serum vs. plasma [7,8]. Two PIs of similar structures, PPACK (thrombin specific) and PPACK II (kallikrein specific), were found to significantly inhibit BNP proteolysis. The
lowest concentration of PPACK II used was capable of 100% protection of exogenous BNP after almost 2 days of storage at 4 °C compared to 90% degradation in control tubes without protease inhibitor. At 50 μg/ml, both PPACK and PPACK II were capable of 100% stabilization after 6 days storage. Since PPACK stabilizes BNP equally to PPACK II, it suggests that the anti-proteolytic effect is not specific to a single series of amino acids (Phe–Pro–Arg vs. Phe–Phe–Arg) and that structurally similar inhibitors of this nature will efficiently protect BNP from the action of the target protease(s).

3.3. Irreversible serine protease inhibitors

Evaluation of generic serine protease inhibitors is shown in Fig. 4. All were tested at concentrations at the upper end of their effective concentration ranges. Aprotinin (100 μg/ml) and benzamidine (15 μmol/ml) have been previously reported as effective inhibitors of BNP proteolysis in blood samples [11]. In the present study, aprotinin was inferior to both arginine aldehyde and kallikrein inhibitors demonstrating only 20% retention of BNP immunoreactivity after 6 days storage. Benzamidine and AEBSF (0.5 mg/ml) prevented about 80% of BNP hydrolysis. Of the two nonpeptide inhibitors, only DFP (0.5% v/v) was highly effective in inhibiting proteolysis.
3.4. Association of BNP proteolytic activity with human serum albumin

In preliminary experiments (data not shown), we observed an association between BNP proteolysis and HSA. The most common method of HSA isolation in bulk quantity is based on the Cohn cold ethanol fractionation technique. Kallikrein isolation also utilizes this fractionation procedure as a starting point for further purification [18]. It was hypothesized that BNP degradation observed when mixed with buffers containing only HSA as a protein might be due to contamination of commercial HSA preparations with kallikrein or kallikrein-like activity. Results presented in the Table 1 suggest the presence of HSA-associated BNP proteolysis in plasma. Both PPACK II and DFP effectively inhibited this activity for up to 4 weeks. The stability pattern of BNP in HSA-containing buffer was similar to storage in plasma, with BNP degradation rates being comparable and both being inhibited similarly with PI. Even in plasma both PPACK II and DFP, at their maximal concentrations, were able to inhibit proteolysis for up to 4 weeks when stored at 4 °C.

3.5. Characterization of a stable protease inhibitor additive

As shown in Fig. 5, after addition of PPACK II to synthetic BNP spiked into human plasma, there was no difference in recovery of BNP after 2 weeks of storage at 4 °C, regardless of the number of freeze-thaws or duration of refrigerated storage of the PPACK II stock solution. Recoveries under all conditions ranged from 89% to 106%. These results demonstrate that, in practice, a stable stock solution of PPACK may be prepared and, under proper storage conditions, can be utilized to stabilize BNP in patient samples for subsequent storage for at least 2 weeks at 4 °C.

Table 1

<table>
<thead>
<tr>
<th>Condition recovery</th>
<th>None (pg/ml)</th>
<th>3 days (pg/ml)</th>
<th>1 week (pg/ml)</th>
<th>2 weeks (pg/ml)</th>
<th>4 weeks (pg/ml)</th>
<th>Final (% of the first run)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control blank buffer (no BNP)</td>
<td>2.6</td>
<td>1.8</td>
<td>1.8</td>
<td>1.2</td>
<td>1.6</td>
<td>–</td>
</tr>
<tr>
<td>Buffer with synthetic BNP without inhibitor</td>
<td>732</td>
<td>120</td>
<td>17.9</td>
<td>2.0</td>
<td>2.6</td>
<td>0.4</td>
</tr>
<tr>
<td>Buffer with synthetic BNP and PPACK II</td>
<td>954</td>
<td>937</td>
<td>903</td>
<td>896</td>
<td>950</td>
<td>99.5</td>
</tr>
<tr>
<td>Buffer with synthetic BNP and DFP</td>
<td>919</td>
<td>899</td>
<td>967</td>
<td>913</td>
<td>930</td>
<td>101.2</td>
</tr>
<tr>
<td>Control blank plasma</td>
<td>11.5</td>
<td>8.7</td>
<td>8.3</td>
<td>7.2</td>
<td>5.9</td>
<td>51.3</td>
</tr>
<tr>
<td>Plasma with synthetic BNP no inhibitor</td>
<td>570</td>
<td>219</td>
<td>73</td>
<td>20.9</td>
<td>8.0</td>
<td>1.4</td>
</tr>
<tr>
<td>Plasma with synthetic BNP and PPACK II</td>
<td>774</td>
<td>747</td>
<td>704</td>
<td>688</td>
<td>714</td>
<td>96</td>
</tr>
<tr>
<td>Plasma with synthetic BNP and DFP</td>
<td>678</td>
<td>736</td>
<td>719</td>
<td>655</td>
<td>705</td>
<td>104</td>
</tr>
</tbody>
</table>
3.6. Stabilization of endogenous BNP in patient samples with PPACK II

To confirm the capability of PPACK II in preventing endogenous BNP degradation, BNP from 10 freshly collected patient plasmas (BNP concentration 12–2800 pg/ml) were stored at room temperature or 2–8 °C and tested at various times for periods up to 3 days in order to develop a temperature stability profile and to assess the impact of PPACK II on preventing BNP decay. As shown in Table 2, the percentage of BNP recovery compared to baseline BNP values measured immediately after samples were drawn demonstrated 24 h stability at 2–8 °C (91% recovery) with progressive degradation between 48 and 72 h. With the addition of PPACK II at the time of sample collection, the average BNP recovery was 99% at 72 h compared to only 67% without the addition of PPACK II.

Based on these findings, an additional series of patient samples were tested out to 6 days in order to approach the maximum stability interval of 7–14 days as suggested by the exogenous BNP spiking experiments (Fig. 5). Results shown in Fig. 6 obtained

![Fig. 5](image-url)  
**Fig. 5.** PPACK II stock solution stability. (A) PPACK II solution was frozen immediately. After up to three freeze–thaws, PPACK II solution was added to plasma samples containing BNP and stored for up to 2 weeks at 4 °C. (B) Same as (A), but PPACK II solutions was stored for 1 week at 4 °C prior to initiation of the study.

![Table 2](image-url)  
**Table 2**  
Stability of endogenous BNP in patient samples with and without PPACK II

<table>
<thead>
<tr>
<th>BNP stability (% recovery)</th>
<th>No inhibitor</th>
<th>No inhibitor</th>
<th>No inhibitor</th>
<th>Without/with inhibitor</th>
</tr>
</thead>
<tbody>
<tr>
<td>Time 0 BNP</td>
<td>2–8 °C</td>
<td>2–8 °C</td>
<td>2–8 °C</td>
<td>2–8 °C</td>
</tr>
<tr>
<td>pg/ml</td>
<td>RT</td>
<td>6 h</td>
<td>24 h</td>
<td>48 h</td>
</tr>
<tr>
<td>1579</td>
<td>96</td>
<td>95</td>
<td>88</td>
<td>70</td>
</tr>
<tr>
<td>628</td>
<td>95</td>
<td>98</td>
<td>82</td>
<td>88</td>
</tr>
<tr>
<td>1269</td>
<td>94</td>
<td>99</td>
<td>82</td>
<td>97</td>
</tr>
<tr>
<td>1601</td>
<td>100</td>
<td>98</td>
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<td>100</td>
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<td>466</td>
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<td>3017</td>
<td>84</td>
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<td>93</td>
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<td>2822</td>
<td>89</td>
<td>92</td>
<td>77</td>
<td>85</td>
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<tr>
<td>645</td>
<td>99</td>
<td>99</td>
<td>85</td>
<td>93</td>
</tr>
<tr>
<td>12.0</td>
<td>80</td>
<td>90</td>
<td>61</td>
<td>110</td>
</tr>
<tr>
<td>Mean recovery</td>
<td>92</td>
<td>95</td>
<td>81</td>
<td>91</td>
</tr>
</tbody>
</table>

**Mean recovery**

92 95 81 91 67 73 67 100

![Fig. 6](image-url)  
**Fig. 6.** Effect of PPACK on stabilization of endogenous BNP in human plasma samples. Patients without inhibitor: (closed symbols); patient with inhibitor (open symbol).
with five additional patient samples confirm that PPACK II stabilizes BNP in patient plasma for at least 6 days. Mean recoveries by 6 days approached >99% with PPACK II, but only 5% in the absence of PPACK II.

3.7. Preliminary analysis of epitopes defined by Shionogi/Centaur and Biosite antibodies

In a previous report, Shimizu et al. [21] developed a preliminary epitope map of BNP-32 by measuring the ability of truncated peptides to bind in the Shionoria RIA. Six peptides were assessed and compared to the cross reactivity of BNP-1–32. Results of this study indicated the epitope recognized by the C-terminal antibody (BC-203) was no longer recognizable when the C-terminal histidine residue alone or in conjunction with three other residues (Arg–Arg–Leu) was removed (BNP 1–31, BNP 1–30, BNP 1–27). In contrast, removal of nine residues from the N terminus (BNP 10–32) had no effect on “sandwich” formation in the Shionoria assay confirming that the antibody pair used in both the Shionoria and Centaur assays is directed against the C terminus (BC-203) and the ring structure (KYhBNP-II). When measured in the Biosite Triage assay, intact peptide BNP 1–32 and fragments 10–32 and 1–27 presented an entirely different reactivity profile. As shown in Fig. 7, compared to intact BNP 1–32, BNP 10–32 had no reactivity in the Triage assay, suggesting that at least one epitope in Triage is directed against the N terminus. Peptide BNP 1–27 had approximately 70% of the reactivity of intact BNP in the Triage test, suggesting that the second antibody used in this assay has reactivity against at least part of the BNP ring and arm which extends to the C terminus. The fact that removal of five amino acids from the C terminus reduces but does not eliminate immunoreactivity suggests that the epitope recognized by the second antibody in the Triage assay requires an extended C-terminal arm in order to be fully reactive.

4. Discussion

It has been widely reported that BNP is metabolized by three separate mechanisms including uptake by natriuretic peptide receptors, renal clearance and enzymatic cleavage by NEPs [1,12]. NEPs (EC 3.4.24.11) are membrane-bound Zn$^{2+}$-containing glycoprotein metalloproteases with a wide tissue distribution. While NEPs are involved in natriuretic peptide inactivation in vivo, the degradation of BNP seen in vitro after sample collection is most likely not due to NEPs for the following reasons. Due to its Zn dependence, NEPs are subject to deactivation by metal-chelating agents, specifically by EDTA [13]. Plasma samples tested in our studies contained EDTA which had no preventative effect on BNP degradation. In addition, it has been reported that NEPs are not inhibited by serine protease inhibitors such as leupeptin, antipain and DFP [13]. In the

![Fig. 7. Reactivity of BNP 10–32 (truncated N terminus) and BNP 1–27 (truncated C terminus) peptide in the Shionoria/Centaur and Triage BNP assays. Shaded bar: BNP 1–32, black bar: 10–32, open bar: BNP 1–27.](image-url)
studies described in this report, all three compounds were capable of inhibiting proteolytic degradation by up to 100%. Finally, we found that some of the most effective in vitro inhibitors of BNP all contain arginine residues (leupeptin, antipain, PPACK I, PPACK II), while a detailed analysis of NEP EC 3.4.24.11 indicates that the preferential cleavage site for BNP is at the Met 4–Val5 bond [20] and at Gly–Phe bond for general peptide cleavage [10].

Shimizu et al [8] first observed that collection and storage of BNP in plastic (polyethylene terephthalate, PET) vs. glass tubes resulted in increased recovery of immunoreactive BNP even when aprotinin was added to the glass tube. They concluded that addition of aprotinin to the PET tube offered no advantage in BNP recovery vs. collection in plastic alone. Our findings of an 80% loss in activity after 6 days storage of aprotinin-containing samples at 4°C corroborate this report. This finding confirms the reports of others that aprotinin is ineffective as an inhibitor of BNP degradation [19].

The enhanced degradation of BNP when collected in glass vials and the increased degradation of BNP in serum compared to plasma led some investigators [7] to consider that the source of proteolytic activity may be due to contact activation of kallikreins via the extrinsic pathway of clotting induced when blood contacts negatively charged surfaces and factor XII is activated. In that study, the authors demonstrated increased kallikrein activity in glass compared to plastic tubes. Since kallikreins are endopeptidases that split peptide substrates preferentially on the C-terminal side of internal arginyl and lysyl residues, this led us to hypothesize that kallikrein-specific inhibitors containing arginine residues may effectively inhibit in vitro degradation of BNP.

As shown in Fig. 1, there are four arginine residues in BNP, two at the C terminus, one at the center of the ring structure and one located at the far end of the ring structure toward the N terminus. Cleavage at any of these sites could destroy BNP epitopes that various antibodies are directed against. For example, in the Shionoria method, studies using synthetic peptides have shown that loss of the C-terminal histidine alone is sufficient to eliminate BNP detection by this assay. Peptides missing one, two, or four residues from the C terminus were also unreactive [21]. This region includes two sites of possible cleavage at Arg-30 and Arg-31. However, loss of up to nine N-terminal residues had no effect in the Shionoria, as the N terminus is not necessary for BNP detection by antibodies employed in this method. The antibodies used in Shionoria and the ADVIA Centaur BNP assay recognize the same epitopes defined by amino acids 27–31 at the C terminus and amino acids 14–21 in the ring structure.

At least one of the epitopes recognized by the Biosite® Triage® assay is located in the N-terminal region, based on a description of antibodies and epitopes disclosed by Scios (Sunnyvale, CA) in patents licensed by Biosite. These include N-terminal regions 1–10 and 5–13 [22]. Cleavage at Arg-13 would compromise either of these epitopes. The studies reported here with BNP peptides, based on the nonreactivity of BNP fragment 10–32, confirm this. Both assays use second antibodies which are directed against epitopes in the ring portion of the molecule [15,17,23].

Chromatographic fractionation and analysis of plasma from heart failure patients (collected in siliconized tubes in order to avoid contact activation of kallikreins) has suggested that prior to any in vitro activation of proteases, there is a natural in situ activity that results in in vivo cleavage of two amino acids at the N terminus (Ser–Pro) [21]. Since limited degradation is observed after 24 h incubation in whole blood at room temperature when testing with assays that recognize the C terminus, it is possible that assays with antibodies that recognize N-terminal epitopes may not recognize this fraction of the total BNP available. This may be one explanation why in our studies, even without protease inhibitor addition, the use of antibodies which recognize C-terminal and ring structure epitopes results in BNP that is more stable in whole blood and plasma under refrigeration than assays that measure BNP using an antibody directed against the N terminus (whole blood: stable up to 24 h at 22°C with C-terminal antibody vs. up to 4 h with N-terminal antibody; plasma: stable up to 24 h at 4°C with C-terminal antibody vs. plasma that must be frozen within 4 h of collection with N-terminal antibody) [10,14,16,21,24]. This would be consistent with the N-terminal residue being the more labile.

The stability of BNP in plasma is markedly improved in the presence of an array of broad spectrum
serine protease inhibitors, especially the more specific kallikrein-related inhibitors of PPACK-like structure. These inhibitors have demonstrated effective protection of both endogenous and synthetic forms of BNP from proteolysis in plasma beyond the 24–48 h seen in their absence. Based on the present report, addition of specific PIs may extend sample stability for up to 10 days.

References