Background. Atrial natriuretic peptide (ANP) is a hormonal regulator of cardiovascular fluid volume. More than 1,000 scientific articles were written about ANP between 1987 and 1991. Because some articles hinted at problems with storing ANP, this study examined the effect of numerous techniques for storing and processing human ANP samples.

Methods and Results. Samples were obtained repeatedly from three patients, treated, and stored under a variety of conditions. Experiment 1 evaluated the effects of different preservatives at 35, 21, 14, 10, and 7 days before assay. Experiment 2 evaluated nonspecific binding of ANP to different storage tubes during 28 days of storage. Experiment 3 evaluated the effect of storage at −20°C, −80°C, and −196°C for 1 month. ANP was very unstable, degrading as much as 30% after 3 days of storage and by more than 50% in 1 month even when stored at −80°C. Only storage at −196°C (in liquid nitrogen) kept ANP stable for 1 month. Extraction and lyophilization of the samples before freezing and assay within 7 days of freezing only partially minimized the amount of degradation. All other processing techniques had little effect on slowing the degradation of ANP.

Conclusions. These findings raise disturbing questions about the interpretation of the substantial literature on ANP. (Circulation 1992;86:463–466)

KEY WORDS • atrial natriuretic peptide • stability • storage

When atrial natriuretic peptide (ANP) was first discovered in the wall of the atria, the finding triggered great scientific excitement.1 Between 1987 and 1991, more than 1,000 articles were published on this fascinating compound, which acts as a hormonal regulator of cardiovascular fluid volume. The literature suggests that ANP levels are altered by illness.2–4 Because of the extremely rapid development of this field and despite the extensive literature, it is vitally important to reexamine the issue of the stability of ANP. To yield valid measures, how should blood samples be processed and stored?

Reports concerning the effect of storage on immunoreactive ANP concentrations are scarce and inconsistent, suggesting very different time ranges before significant breakdown in ANP.5–9 Various reports have suggested that ANP levels remain stable from as little as 3 days to as long as 6 months. Furthermore, these studies have examined ANP levels from normal volunteers, whose ANP concentrations are so low as to be near the sensitivity limits of the assay.8,9

The purpose of this study was to examine the effects of different sample collection, processing, and storage techniques on ANP stability.

Methods

Samples

Samples were collected from three patients with renal failure undergoing hemodialysis who signed a written informed consent that was approved by the Institutional Review Board of the University of California San Diego. All samples were obtained just before the scheduled hemodialysis and at the same time of day. There were no changes in medication or interdialysis weight gain during the interval of repeat sampling. We also obtained samples from pooled plasma, collected from normal subjects, with no detectable ANP activity; to these samples we added 200 pg/ml ANP (Sigma) each day blood was drawn from the patients. Samples were collected 35, 21, 14, 11, and 7 days before the assay, and reference samples were obtained on the day of assay.

Sample Stabilization

On every collection day, the four samples (three patients and one spiked, pooled plasma) were aliquoted and treated with the five following additive and storage regimens: 1) Blood was collected into heparinized tubes and stored at −80°C; 2) blood was collected into EDTA (1 mg/ml blood) and stored at −20°C; 3) blood was collected into EDTA and stored at −80°C; 4) aprotinin (500 KIU/ml plasma) was added to the EDTA-treated plasma stored at −80°C; and 5) aprotinin-treated plasma was extracted through a solid-phase extraction column and stored at −80°C.
In experiment 1, every 2-ml plasma sample was acidified with 2 ml of 0.1% trifluoroacetic acid (TFA) before extraction or freezing and stored in polypropylene test tubes. All samples except the reference samples were extracted and lyophilized 1 week before assay. Reference samples were drawn, extracted, and lyophilized on the day of assay.

Experiment 2 evaluated the possibility of nonspecific binding to the collection or storage tubes. Samples were collected 28 days before assay with EDTA (1 mg/ml) and stored at −80°C in four different types of test tubes: polypropylene, polystyrene, silanized glass, or glass tubes. A fifth set of samples was treated with Triton-X (Calbiochem) 0.1% before extraction and stored in polypropylene tubes at −80°C. A sixth set of samples was stored at −20°C in polypropylene tubes. All samples in this experiment were acidified, extracted, and lyophilized before storage and were kept in their original collection tube conditions throughout the assay procedure from collection through gamma counting. Reference samples were drawn, extracted, and lyophilized on the day of assay.

Experiment 3 studied the effects of acidification before storage as well as storage at −196°C. Blood samples were collected with EDTA (1 mg/ml). The plasma was treated in the following four ways: 1) addition of 0.1% TFA; 2) addition of apoprotinin (500 KIU/ml plasma); 3) addition of TFA and apoprotinin; and 4) no additives. These plasma samples were stored for 1 month at −196°C (liquid nitrogen), at −80°C, and at −20°C. Reference samples were drawn and treated as above on the day of assay. All samples were extracted and lyophilized before assay.

Radioimmunoassay was performed with the commercially available Alpha Human Atrial Natriuretic Polypeptide Kit.10

Results

All of the ANP samples in experiment 1 degraded over time (Figure 1). The samples collected with EDTA and stored at −80°C had less breakdown than the samples collected with heparin or those stored at −20°C. Adding a protease inhibitor or extracting the samples before storage at −80°C did not improve ANP stability beyond that of the samples collected with EDTA alone. Heparin was the worst medium for treatment of the samples; such samples lost about 50% of their ANP immunoreactivity before lyophilization and >70% of their activity after 3 days of storage. Storage at −20°C produced similar results. Ly-
ophilization of the extracted ANP, however, resulted in relative stability for 7 days (<10% breakdown).

The substantial decay in ANP levels was not a function of the collection tube material. Storage in tubes other than polypropylene for 1 month also resulted in a large reduction in ANP concentration (Figure 2). Collecting and storing the samples in polystyrene or storage at -20°C resulted in the largest reduction of ANP activity. Although collection into polypropylene, silanized glass, or glass and treatment with Triton-X resulted in smaller amounts of degradation, the amount lost remained sizable. Thus, collection and storage of plasma in any type of tube plus extraction and lyophilization of the samples before 28 days' storage resulted in >50% degradation of the ANP levels.

Storage in liquid nitrogen resulted in acceptable stability of the ANP samples for 1 month (Figure 3). No sample deterioration was noted in these samples relative to the reference samples. Storage at -80°C and -20°C resulted in sizable breakdown of ANP. Treatment of the samples with apoprotinin and/or acid before storage had no effect on ANP stability.

To exclude the possibility that renal failure may increase the breakdown of ANP, we compared the percent change in ANP levels in the patients with renal failure with the percent change in level from pooled spiked plasma from normal subjects. Compared with the renal patients, the spiked plasma levels showed similar degradation.

**Discussion**

Plasma ANP degraded by >50% in 1 month under typical storage conditions. Surprisingly, ANP extracted on a Sep-Pak cartridge and lyophilized to dryness and stored at -80°C still deteriorated. Only sample storage in liquid nitrogen resulted in the ANP samples' remaining stable for at least 1 month.

One study suggested that ANP from normal subjects is stable for up to 6 months if it is treated with apoprotinin, stored at -80°C, and thawed once after collection. Normal subjects have such low basal ANP levels that it is difficult to appreciate sample deterioration, given the sensitivity of the assay at such low levels. For this reason, we used samples from patients with renal failure before their hemodialysis, because such patients have high ANP levels. The high ANP levels in these samples permit a clear appreciation of ANP degradation. We also used plasma spiked with ANP to exclude the possibility that our results might be biased by ANP breakdown products in the circulation of patients with renal failure. Nonspecific binding of ANP to the collection or storage tubes did not appear to be the cause of the loss of activity, because similar breakdown occurred with all collection techniques and collection media.

We measured ANP with the most frequently used commercial assay for this peptide. Saxenhofer et al. demonstrated by reverse-phase high performance liquid chromatography that this assay measures authentic ANP in plasma from dialysis patients. Furthermore, normal plasma, without any detectable ANP immunoreactivity, spiked with synthetic ANP had a decay of ANP immunoreactivity indistinguishable from the decay in the plasma of dialysis patients.

These are disturbing findings for a field that has grown so swiftly. The findings of this study suggest that the literature on human ANP levels may be unreliable unless details of sample storage are provided. Plasma samples for ANP levels should be anticoagulated with EDTA and assayed immediately, stored for only a few days at -80°C, or stored in liquid nitrogen for longer periods. Samples should not be stored in polystyrene or glass tubes. There are hundreds of published studies of plasma ANP; most do not report how samples were stored. Degradation of ANP during storage could explain discrepancies between ANP levels reported in different studies.

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