Concentrations of atrial natriuretic peptide (ANP) are increased in plasma of patients with impaired cardiac and renal function. The second messenger of ANP, cyclic guanosine monophosphate (cGMP), is released into the plasma specifically upon stimulation of cells with ANP. Although nitrates can also activate intracellular cGMP synthesis, we detected no increase in plasma cGMP concentrations after infusions of glycerol trinitrate. Because immunoreactive ANP is highly susceptible to degradation and nonspecific influences in blood samples, determinations of ANP require immediate centrifugation and storage of plasma at -20 °C. In contrast, we found that cGMP is stable for five days in vitro in blood samples containing EDTA. In 147 healthy blood donors, the upper cutoff value for plasma cGMP was 6.60 nmol/L, not significantly different (P > 0.05) from that for 222 patients with disorders other than cardiovascular and renal. In 69 patients with manifest congestive heart failure (NYHA stages II-IV), 65 had increased cGMP values. Using the above cutoff value for cGMP gave diagnostic sensitivity of 94.2% and specificity of 93.7%. Plasma cGMP may thus provide an alternative for routine clinical measurements of ANP in cardiac diseases in the absence of renal disorders.

Additional Keyphrases: atrial natriuretic peptide  •  renal function  •  cutoff value  •  nitrates  •  radioimmunoassay

Atrial natriuretic peptide (ANP) has been the subject of intensive physiological and clinical research over the past few years. So far, however, this considerable amount of research does not seem to have benefited clinical diagnostics.

Increased concentrations of ANP in peripheral blood are detectable in patients with tachyarrhythmias (1-4), with congestive heart failure (4-6), and after volume load (7-9). In addition, an increase in ANP concentrations in plasma can be caused by impaired elimination of the peptide in chronic renal failure (9-11). Increases in atrial pressure are a well-known and important stimulus for ANP release from atrial cardiomyocytes (12). The sympathetic nervous system also plays a role in the regulation of ANP secretion and in mediating the effects of ANP on target cells (13).

The physiological signal given by release of ANP into the blood is transmitted intracellularly by cyclic guanosine monophosphate (cGMP) (14-16). Interestingly, release of cGMP from cultured cells into the medium has been observed after stimulation with the hormone (17). Also, high concentrations of ANP in plasma in vivo cause an increase in cGMP concentrations in plasma (18, 19). In patients with heart diseases, good correlations have been found between cGMP and increased ANP concentrations and with clinically assessed severity of congestive heart failure (5).

Several reports about ANP concentrations in plasma from patients with impaired cardiovascular function have been published (1-9). The purpose of the present study was to evaluate the significance of the ANP-cGMP system for laboratory diagnosis of cardiac diseases, and to assess whether such information would be applicable in the routine laboratory. Among the pre-analytical and analytical difficulties posed by ANP are the temporal fluctuations in ANP concentrations detected by radioimmunoassay in vitro, possibly caused by specific and nonspecific proteolysis and reversible interactions of the free peptide with the vial, which might influence the structure or the accessibility of the epitopes recognized by an antibody (20). Therefore, an immediate determination of ANP after blood withdrawal, or immediate centrifugation and freezing of the plasma sample are necessary to obtain reliable results. In addition, several different physiological cleavage products of ANP are present in human plasma (20, 21). This makes difficult the assessment of reproducible reference values, which thus far depend on the specificity of the antibody used in the radioimmunoassay. In fact, Genest and Cantin (20) reported considerable differences in the reference intervals proposed by several authors, even when similar methods of extraction or determination of plasma ANP concentrations were used. In contrast, cGMP—being a nucleotide—is not as susceptible as peptides to nonspecific influences in vitro.

Two isoenzymes of guanylate cyclase are responsible for cGMP production (22): a soluble form, which can be stimulated by organic nitrates such as glycerol trinitrate (GTN) or sodium nitroprusside (23, 24), and a particulate form, which represents the intracellular domain of a transmembranous protein carrying an ANP receptor on the external surface of the membrane (25). The only substances known to activate the particulate
guanylate cyclase are ANP (26) and a heat-stable enterotoxin from *Escherichia coli* (27). Given our knowledge of these different activators, the major objection to determining plasma cGMP for diagnostic purposes has been its uncertain specificity for ANP. Gerzer et al. (19) were able to detect a distinct increase in plasma cGMP concentrations after an intravenous bolus of ANP in healthy subjects. Hauptlorenz et al. (28) described the course of ANP and cGMP concentrations in plasma of patients with acute myocardial infarction, finding an increase of both substances shortly after the onset of symptoms. Despite the infusion of high doses of GTN for several hours after admission, plasma cGMP concentrations decreased to normal values, in parallel with a decrease in ANP concentrations. That the release of cGMP into the plasma is specific for stimulation of cells with ANP is supported by recent experiments (29) showing an ANP-specific extrusion of cGMP from isolated aorta into the medium, whereas nitroprusside had no effect on the release of cGMP.

Encouraged by these results, we evaluated the specificity, sensitivity, and clinical efficiency of cGMP as a marker of diseases associated with increased concentrations of ANP in plasma, particularly congestive heart failure.

### Materials and Methods

#### Stability of cGMP

Blood was drawn from the cubital vein of healthy volunteers into tubes coated with EDTA (1.5 mg/mL of blood), sodium citrate (0.106 mmol/mL), or lithium heparin (15 units/mL), or without any additives, and centrifuged without delay. The plasma was divided into two portions and stored at room temperature or at 4 °C. After various intervals (2–10 min for determination of half-life, 5–24 h for determination of stability), 250-μL aliquots were frozen at −20 °C.

#### Patients and Healthy Volunteers

To establish reference values for cGMP, we took blood samples from 147 healthy volunteers (55 females, 92 males; ages 15–62, mean ± SD = 34 ± 13 years) who were not receiving any drug therapy, and whose blood pressures did not exceed 180/100 mmHg. To exclude persons with renal impairment, we determined plasma creatinine concentrations in all subjects, and excluded from the study those whose values exceeded 125 μmol/L. A group of hospitalized patients with other than cardiovascular and renal diseases consisted of 222 persons (117 women, 105 men; ages 17–79, mean ± SD = 46 ± 16 years) categorized in nine different disease groups: diabetes mellitus (n = 22; 49 ± 15 years), pulmonary diseases (n = 17; 45 ± 13 years), infectious diseases (n = 23; 36 ± 16 years), gastroenterologic diseases (n = 7; 32 ± 15 years), hepatologic diseases (n = 43; 43 ± 13 years), rheumatologic diseases (n = 13; 52 ± 16 years), psychiatric diseases (n = 22; 46 ± 13 years), neurologic diseases (n = 36; 44 ± 17 years), and hematologic neoplasias (n = 39; 56 ± 13 years). Patients taking antihypertensive drugs were excluded from this study, as were those whose blood pressures exceeded 180/100 mmHg at the time of blood sample collection. Twelve further patients (three women, nine men; ages 26–61, mean ± SD = 41 ± 14 years) with renal failure (n = 9; 41 ± 14 years) or glomerulonephritis (n = 3; 40 ± 19 years) formed the group of patients with renal diseases. All of these subjects had increased concentrations of creatinine in plasma (range 186–652 μmol/L, mean ± SD = 319 ± 160). Five additional patients with both cardiac and renal failure were excluded.

Diagnostic sensitivity was determined in a group of 69 patients (16 women, 53 men; ages 20–76, mean ± SD = 57 ± 10 years) from the Intensive Care Unit of the Department of Internal Medicine, University Hospital of Innsbruck, Austria, and from the Rehabilitation Center in Grossmain, Salzburg, Austria, who had manifest congestive heart failure [New York Heart Association (NYHA) stages II–IV] at the time of blood sampling. This group comprised 37 patients with congestive heart failure after myocardial infarction (mean age ± SD = 59 ± 9 years), 15 with coronary heart diseases (57 ± 7 years), eight with valvular diseases (53 ± 15 years), seven with cardiomyopathy (48 ± 9 years), one with a sinusatrial block (56 years) and one with congestive heart failure of unknown origin (60 years). The criteria for exclusion were the same as mentioned above (hypertension, antihypertensive drug therapy, and plasma creatinine concentrations exceeding 125 μmol/L).

All blood samples were collected into EDTA-coated tubes (1.5 g/L of blood). After centrifugation for 10 min at room temperature, plasma was stored at −20 °C until assayed.

#### Effect of ANP and GTN on cGMP in Plasma

Two healthy volunteers (one 24-year-old man, 80 kg; one 22-year-old woman, 52 kg) each underwent two experiments. In all four experiments, blood pressure and heart rate were monitored at 1-min intervals. After centrifugation, the plasma obtained was stored at −20 °C.

**ANP bolus:** After a resting period of 20 min, both subjects received a bolus of ANP, 0.625 μg/kg, into the cubital vein. Blood samples were drawn from the contralateral cubital vein into EDTA-coated tubes in 2–10-min intervals during 20 min before and 30 min after the bolus. The synthetic human ANP was purchased from Bissendorf Peptide (Wedemark, F.R.G.), and dissolved in isotonic saline (NaCl 150 mmol/L) to a final concentration of 50 mg/L.

**GTN infusion:** After the same resting period, an infusion of 50 mL of isotonic saline containing 10 mg of GTN was started, at a rate of 1 mL/min (0.2 mg/min). After each 10-min period, the infusion rate was doubled. Total doses of GTN were 10 mg for the man and 6 mg (infusion stopped due to onset of headache) for the woman. Blood was withdrawn from the ipsilateral cubital vein after rinsing the canula with isotonic saline; 2 mL of blood was drawn and discarded after each rinsing step. The ANP bolus and the GTN infusion were separated by at least one week.

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Analysis

For plasma extraction, 1 mL of ethanol was added to 250 μL of plasma. After centrifugation for 15 min, supernatants were collected, and the pellets were dissolved in 500 μL of ethanol and recentrifuged for 15 min. These supernatants were then combined with the first supernatates and dried at 57 °C under a stream of nitrogen. Extracted samples were reconstituted in 1 mL of assay buffer, and 500 μL was used for analysis.

Concentrations of immunoreactive cGMP were measured with a 125I-labeled radioimmunoassay (cGMP-assay RPA 525; Amersham International, Amersham, Bucks., U.K.). Inter- and intra-assay CVs were 12.2% and 9.2%, respectively. Plasma creatinine concentrations were measured enzymatically with reagents from Boehringer Mannheim (Mannheim, F.R.G.).

Statistical analysis was performed with the Mann–Whitney U-test. P-values <0.05 were considered significant.

Results

Stability of cGMP: In vitro stability of cGMP for 90 h is demonstrated in Figure 1. EDTA specifically inhibits cGMP-degrading phosphodiesterases, whereas the cyclic nucleotide is rapidly degraded in untreated serum, with a half-life between 10 and 20 min. Differences in temperature appear not to influence stability or degradation of cGMP. Figure 1 also illustrates that degradation is not primarily associated with blood coagulation and a possible release of cellular phosphodiesterases, because it can be prevented by neither citrate nor heparin. Immunoreactively detected (ir-) cGMP concentrations in an EDTA plasma sample remain stable for five days, decreasing in activity by 30–50% after 30 days. Storage of EDTA plasma samples at −20 °C prevents decomposition of ir-cGMP for at least six months. Similar results are obtained without immediate centrifugation after blood withdrawal, showing that cGMP is little affected by pre-analytical and analytical handling.

Specificity and sensitivity: For determination of the diagnostic efficiency (number of correct test results corresponding to disease and non-disease) of cGMP in cardiovascular disease, we quantified cGMP in plasma from healthy persons, patients with noncardiovascular diseases, and patients with manifest cardiac diseases. Figure 2 shows the histograms for cGMP concentrations in plasma from healthy blood donors (a), patients without cardiovascular or renal impairment (b), and patients with congestive heart failure (c). Figure 2d compares cumulative frequencies of the three groups. Because of slight deviations from gaussian distribution, we used nonparametric methods throughout (Q1 = lower quartile, Q3 = upper quartile).

In the group of 147 healthy blood donors, the median concentration of cGMP in plasma was 4.50 nmol/L (Q1 = 3.60, Q3 = 5.52 nmol/L) and the 90% interval ranged from 2.24 to 7.20 nmol/L (Figure 2a). In patients with other than cardiovascular or renal diseases, a similar distribution was obtained: median 4.24 nmol/L (Q1 = 3.24; Q3 = 5.20; 90% interval 2.19–6.80 nmol/L) (Figure 2b).

The reference interval for our subjects is higher than that calculated by Wencker et al. (30) for 125 healthy blood donors (2.90 ± 1.45 nmol/L), probably because Wencker et al. used 3H-labeled RIA, whereas we used 125I-labeled RIA.

Plasma cGMP concentrations measured in 69 individuals with cardiac insufficiency ranged from 3.89 to 17.89 nmol/L (Figure 2c). The correlation between cGMP concentrations and NYHA classification of the severity of congestive heart failure described by others (5) could be confirmed. Two patients belonging to NYHA class IV, whose cGMP concentrations were very high (18.4 and 26.12 nmol/L), were excluded because of high plasma creatinine values (263 and 222 μmol/L, respectively) caused by secondary impairment of renal function.

The difference between the distribution of cGMP values in blood donors and patients without cardiovascular and renal diseases is not statistically significant.
In accordance with results of Wencker et al. (30), neither of these groups showed any effect of sex or age on plasma cGMP. The distribution of cGMP concentrations in the different noncardiac disease groups is shown in Figure 3. All 12 patients with chronic renal failure or glomerulonephritis had increased cGMP concentrations in plasma (Figure 3), with a range from 9.27 to 15.24 nmol/L (median 12.80; Q1 = 10.9; Q3 = 14.92 nmol/L). The correlation between plasma cGMP and creatinine concentrations was not significant ($r = 0.202$). Like the other patients with increased concentrations of plasma creatinine, this group was not included in the calculation of specificity.

To determine the upper cGMP cutoff value, we considered two possibilities: the 95th percentile, and the value yielding the highest efficiency ($\text{eff} = (\text{specificity} + \text{sensitivity})/2$) calculated by the maximum of Youden indices ($Y = \text{spec} + \text{sens} - 1$) (31). Figure 4 demonstrates that an upper cutoff value of 6.6 nmol/L calculated by the Youden index for patients without cardiovascular or renal impairment has the greatest efficiency (0.940). For the 95th percentile concentration (6.8 nmol/L), efficiency was lower (0.929). Therefore, we chose the cutoff value of 6.6 nmol/L for our calculations, for which the sensitivity was 94.2% and the specificity was 93.7% for manifest congestive heart failure.

**Effect of ANP and GTN on cGMP in plasma:** Concentrations of cGMP in plasma before and during infusion of GTN and a bolus of ANP are shown in Figure 5. GTN had no significant effect on plasma cGMP, whereas a bolus of ANP markedly increased plasma cGMP concentrations within 2 min.

**Discussion**

The rapid increase in cGMP in plasma after a bolus of ANP, the high diagnostic sensitivity of cGMP for manifest congestive heart failure, and the increased cGMP concentrations in patients with chronic renal diseases imply that increases in plasma cGMP reflect increases in plasma ANP concentrations. Additionally, the results of this study point out the specificity of plasma cGMP for changes in ANP concentrations, which is consistent with the findings of Gerzer et al. (19) and Stasch et al. (29). There is no evidence that the reaction of plasma cGMP to ANP is different in patients with cardiac impairment (30).

Thus far, the exact molecular mechanism of cGMP secretion into plasma after stimulation with ANP is not known. Obviously, it represents a specific reaction stimulated by ANP and provides another mechanism for regulation of intracellular cGMP in addition to cleavage of the nucleotide by phosphodiesterases.

The correlation between ANP and cGMP found by Hirata et al. (5) in the plasma of patients with cardiac diseases ($r = 0.74$) has formerly been interpreted to reflect the lack of specificity of cGMP for ANP. The deviation from a linear correlation is probably caused by a down-regulation of ANP receptors in target cells, with a subsequent relative decrease in cGMP production (32, 33), and by different kinetics and pathways of clearance of both substances: ANP is eliminated by the kidney (34, 35) and by the vascular beds of several organs (36), whereas the only organ known to eliminate cGMP from plasma is the kidney (15).

The diagnosis of manifest severe congestive heart failure does not represent a problem to the clinician.
However, measurements of plasma cGMP could provide a useful tool for monitoring therapeutic effectiveness in congestive heart failure (37). In addition, cGMP could support the diagnosis of mild forms of the disease (NYHA stages I and II): physical exercise under standardized conditions causes an increase in ANP concentrations to pathological values in patients with mild congestive heart failure, whereas in healthy persons ANP concentrations do not exceed the normal range (38, 39). In a preliminary study, we obtained analogous results for cGMP concentrations in plasma.

Finally, we conclude that measurement of plasma cGMP is suitable for routine diagnostic applications, whereas pre-analytical and analytical difficulties can obscure the interpretation of routine determinations of ANP.

We dedicate this paper to Prof. Dr. Herbert Keller (St. Gallen, Switzerland) on his 65th birthday.

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