Short Communication

Proguanylin: development, analytical characterization, and clinical testing of a new ELISA

D. Stejskal[1], P. Solichova[1], B. Lacnak[1], J. Vaclavik[1], Z. Hanulova[2], V. Humenanska[2], L. Sprongl[3] and M. Karpisek[4]

[1] *Department of Laboratory Medicine and Internal Department, Šternberk Hospital, Czech Republc*
[2] *BioVendor – Laboratory Medicine Inc., Modřice, Czech Republic*
[3] *Central Clinical Laboratories, Šumperk Hospital, Šumperk, Czech Republic*
[4] *Faculty of Pharmacy, University of Veterinary and Pharmaceutical Sciences, Brno, Czech Republic*

Abstract. The aim of our work was to develop an assay for the determination of proguanylin in human blood, and investigate its levels in healthy volunteers and donors suffer from hypertension often accompanied by body sodium accumulation and plasma volume expansion. We developed and evaluated the sandwich ELISA method for the quantitative determination of human proguanylin in serum samples. We conducted also the pilot study on individuals with hypertension and oh healthy probands and measured proguanylin serum levels, serum and urine sodium and creatinine levels. In the study on 256 healthy volunteers we demonstrated that women have significantly higher values of proguanylin than men (medians 12.7 *vs*. 9.6 ng/ml, $p < 0.01$) and proguanylin values increased with age of individuals ($p < 0.01$). Futhermore, we tested 17 individuals with hypertension and found that probands with anamnesi of hypertension had higher proguanylin values than healthy individuals from the first study (medians 16.2 *vs*. 11.3 ng/ml, $p < 0.01$).

Both of groups did not differ in sex or age. Proguanylin values correlated with the systolic blood pressure ($r = 0.41$, $p < 0.01$), sodium fraction excretion ($r = 0.72$, $p < 0.01$) and serum sodium ($r = -0.39$, $p < 0.01$). No significant correlation we found with serum proguanylin and creatinine. In the group of 9 healthy probands we demonstrated the existence of a diurnal rhythm of proguanylin with its maximum in the evening hours (between 6–10 p.m.).

The pilot study supports the hypothesis about the role of proguanylin in sodium metabolism and its possible importance for hypertension disorder. Further research is necessary to confirm our findings in individuals with hypertension with different medication in order to assess proguanylin value as a risk predictor of accelerated hypertension, and to classify individuals with hypertension for variuos types of diuretic therapy.

Key words: Proguanylin — Hypertension — Guanylin — ELISA

Electrolyte and water regulation is crucial to the management of fluid volume. The cyclic guanosine monophosphate signal pathway is suggested to be an essential mechanism controlling renal function with respect to urinary excretion of sodium, potassium, and chloride (Drewett and Garbers 1994; DiBona and Kopp 1997). The action of natriuretic peptides, involved in the regulation of salt and water homeostasis (Cogan 1990), is associated with binding on receptor with guanyl cyclase activity. Seven receptors with guanyl cyclase activity have been identified, two of them (CG-A and CG-B) bind natriuretic peptides type a and B, however, ligands for the other receptor have not yet been found out. Guanylin is one of the peptides supposed to bind to the CG-C receptor, controlling intenstinal electrolyte and water transport. Guanylin is an endogenous peptide that was originally isolated from urine and intestine,

Correspondence to: David Stejskal, Department of Laboratory Medicine and Internal Department, Šternberk Hospital, Jivavská 20, 785 16 Šternberk, Czech Republic (www.nemstbk.cz/olm)
E mail: david.stejskal@quick.cz

respectively (Curie et al. 1992; Kita et al. 1994). In humans, the peptide is initially synthesized as preproguanylin, which consists of 115 aminoacids. Cleveage of 21 N-terminal residues leads to formation of 94-aminoacid proguanylin. Mature guanylin requires proteolytic processing into small COOH-terminal 15 aminoacid peptide that binds to and regulates CG-C in kidney and intestinal epithelial cells (Forte et al. 1989; Forte and Hamra 1996; Forte 2004). Guanylin mRNA has been detected above all in the gastrointestinal tract in numerous cell types, including goblet cells, Paneth cells, surface villous enterocytes, enteroendocrine cells. It seems that guanylin has paracrine functions whereas its prohormone might play a role as a link between intestine and kidney (intestinal-renal endocrine axis). Guanylin circulates exclusively as proguanylin, no mature guanylin has been detected in plasma. Plasma proguanylin is freely filtred through glomelural barrier in the kidney. Urine contains no or very low amount of proguanylin probably because of degradation by chymotrypsin-like proteases in the proximal tubule (Arao et al. 1994; Hamra et al. 1996) or in the glomeruli (Greenberg et al. 1997) or can be endocytozed from the lumen in proximal tubules (Furuya et al. 1997). However, proguanylin has been isolated from human serum (Nakazato et al. 1994) and also from concentrated dialysate of patients with end-stage renal disease (Kuhn et al. 1993). Into the intestinal lumen, guanylin inhibits Na^+ absorption and induces Cl^-, HCO_3^-, and water secretion. Simultaneously, that hormone stimulate renal electrolyte excretion by inducing natriuresis and diuresis (Sindic and Schlatter 1994; Forte 2004). Since no relevant information on circulating proguanylin levels has been published, the aim of our work was to develop an assay for the determination of proguanylin in human blood, and investigate its levels in healthy volunteers and donors suffer from hypertension often accompanied by body sodium accumulation and plasma volume expansion.

We established and evaluated the new sandwich ELISA for the determination of human proguanylin in serum samples. The assay employs imunoaffinity purified rabbit polyclonal anti-human proguanylin antibody provided by BioVendor – Laboratory Medicine Inc. (Czech Republic) and coated in microtiter wells (High Binding type, Corning Costar): 100 μl/well, 3 μg/ml in 0.1 mol/l carbonate buffer (pH 9.0) overnight at 4°C. The plate was once washed with TBS-Tw (0.05 mol/l Tris-HCl; 0.15 mol/l NaCl; pH = 7.2; 0.05% (w/v) Tween 20) on the washer Columbus (Tecan, Inc.). Nonspecific binding sites were blocked with 250 μl/well 1% bovine serum albumin (BSA; w/v) in TBS-Tw for 30 min at 25°C. After the aspirating step, the diluted samples (100 μl of human serum or plasma samples diluted 10× with TBS (0.05 mol/l Tris-HCl; 0.15 mol/l NaCl; pH = 7.2); 0.1% BSA (w/v); 0.01% thimerosal) or standards were pipetted in duplicates at 100 μl/well. The plate was incubated for 1 h at 25°C. After 3 times repeated washing step with TBS-Tw, 100 μl/well of horseradish peroxidase-labelled rabbit anti-human proguanylin polyclonal antibody (BioVendor – Laboratory Medicine Inc.) at the concentration 0.5 μg/ml in TBS (pH = 7.2), 0.5% BSA (w/v), 0.01% thimerosal) was added and the plate was incubated for 1 h at 25°C. After 3 times repeated washing step with TBS-Tw, 100 μl/well of tetramethylbenzidine substrate (KPL, Inc.) was added and incubated for 10–15 min at 25° C under dark conditions. Reaction was stopped with 100 μl/well of sulfuric acid (0.2 mol/l). The developed color reaction was determined by reading the plate on the microplate reader MRX II (Dynex, Inc.) at a wavelength of 450 nm. As the standard we used a recombinant proguanylin (BioVendor – Laboratory Medicine Inc.). The protein content was determined by the bicinchoninic acid assay (Sigma-Aldrich, Inc.) and the purity greater than 95% was confirmed by SDS-PAGE (Fig. 1A). Standards were prepared at concentrations of 10, 5, 2.5, 1.25, 0.6 and 0.3 ng/ml in TBS (pH 7.2), 0.1% BSA (w/v), 0.01% thimerosal) and 100 μl directly pipetted into the wells (Fig. 1B). The specificity of

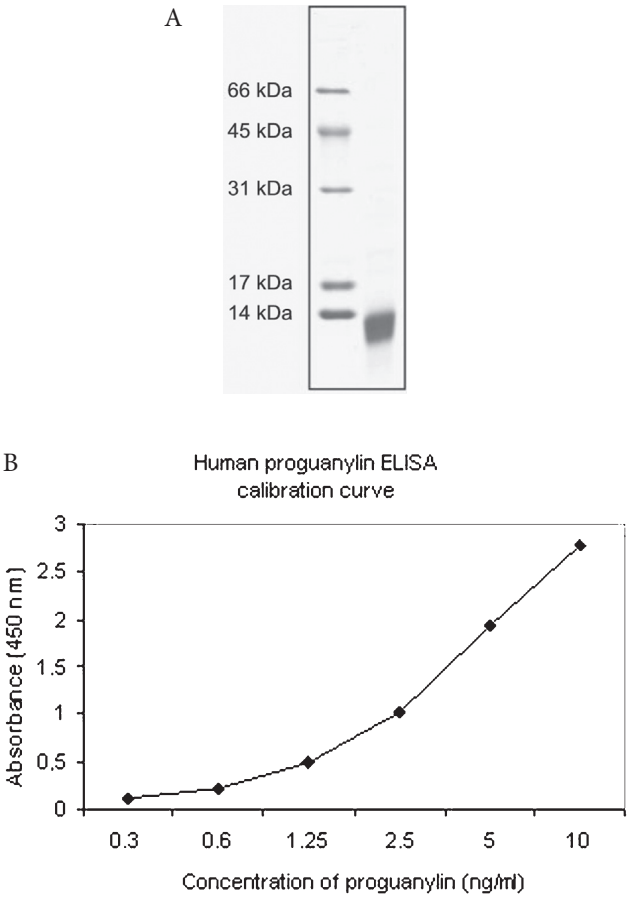

Figure 1. A. Recombinant human proguanylin was analyzed in SDS-PAGE under reducing conditions (12% homogenous gel, Laemli method), 5 μg/lane. **B.** Calibration curve for human proguanylin is plotted as a proportion of proguanylin concentration and absorbance at 450 nm.

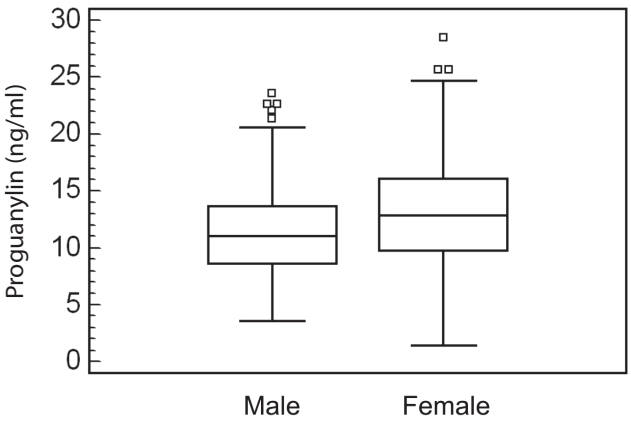

Figure 2. Proguanylin mean values by sex of healthy probands (141 men, 115 women).

the assay was confirmed in the test of cross-reactivity with human recombinant guanylin (AnaSpec, Inc.) and human prouroguanylin (BioVendor – Laboratory Medicine Inc.) when no signal was observed as well as for sera of several mammalian species: mouse, rat, rabbit, horse, cow, sheep, goat and pig. To validate the reliability of the assay, we tested the precision and the accuracy of the assay. To analyze the spiking recovery, human serum sample from two subjects with baseline proguanylin level 0.93 and 1.35 ng/ml were spiked with increasing amounts of recombinant protein (+1.25, +2.5 and +5 ng/ml) and assayed. The mean recovery was 94%. Moreover, we tested human serum samples from another two subjects with baseline proguanylin level 2.58 and 3.27 ng/ml in dilution linearity. The mean recovery was 96%. The limit of detection of the assay was 0.09 ng/ml, intraassay and interassay coefficient of variation were 6.7% and 8.3%, respectively.

Our pilot study involved the group of heathy volunteers, and the group of donors suffering from hypertension. We determined proguanylin levels by using ELISA presented above. The study was arranged as follows: in the study on healthy volunteers (n = 256) we demonstrated that women have significantly higher values of proguanylin than men (abnormal distribution, medians 12.7 *vs.* 9.6 ng/ml, $p < 0.01$, sampling in the morning after 8 h of fasting) (Tab. 1). We also found that proguanylin values increased with age of individuals (not shown) ($p < 0.01$). Furthermore, we tested 117 individuals with hypertension (1st grade by WHO classification), treated only inhibitors of angiotensine converting enzyme and acetylsalicylic acid (100 mg *per* day). In addition to proguanylin levels, we determined blood pressure and the quantity of excreted sodium. Probands with anamnesis of hypertension had higher proguanylin levels than healthy individuals from the first group (abnormal distribution, medians 16.2 *vs.* 11.3 ng/ml, $p < 0.01$) (Tab. 2). Both of groups did not differ in sex or age (Fig. 2). Proguanylin correlated with the systolic blood pressure ($r = 0.41$, $p < 0.01$), natriuresis ($r = 0.72$, $p < 0.01$) and serum sodium ($r = -0.39$, $p < 0.01$) (not shown). No significant correlation was found between serum proguanylin and creatinine levels (not shown). Therefore we believe that proguanylin might be in relation to salt dependent type of hypertension.

Table 1. Distribution of proguanylin (ng/ml) by sex of tested individuals

Group	Mean	Median	SD	Normality
Women	21.4	12.7	8.4	–
Men	12.1	9.6	6.2	–

Levels of serum proguanylin were measured in 141 men and 115 women of matching age according to the method described in the text, $p < 0.01$. Normality tested by Komolgorov–Smirnov. SD, standard deviation.

Table 2. Proguanylin (ng/ml) in individuals with hypertension (+HYP) and without hypertension (–HYP)

Group	Mean	Median	SD	Normality
+HYP	27.7	16.2	19.9	–
–HYP	15.3	11.3	10.1	–

Levels of serum proguanylin were measured in 117 patients with hypertension and 156 healthy volunteers according to the method described in the text, $p < 0.01$. Normality tested by Komolgorov–Smirnov. SD, standard deviation.

Table 3. Diurnal rhytmicity of serum proguanylin (ng/ml) in 9 healthy probands

Hours	Mean	Median	SD
08:00	6.1	8.2	4.1
10:00	10.2	9.2	3.7
12:00	9.4	8.2	3.1
14:00	9.1	8.7	2.1
16:00	11.0	9.6	3.2
18:00	12.1	10.0	4.1
20:00	12.3	11.2	5.1
22:00	11.9	11.0	4.1
24:00	13.0	10.0	5.0
02:00	10.0	9.4	3.2
04:00	7.9	8.6	4.1
06:00	8.3	8.1	2.2

Blood was withdrawn at time of day shown in the table and serum proguanylin levels were determined as described in the text. SD, standard deviation.

In the group of 9 healthy probands, we demonstrated the presence of diurnal rhythm of proguanylin with the maximum levels in evening hours (between 6–12 p.m.) (Tab. 3).

In summary, presented results demostrated the analytical competence of the ELISA guanylin assay and showed its usefulness for the study of hypertension.

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Final version accepted: November 2, 2006