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Molecular components and concentrations of human serum prorenin

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Objectives: Human plasma prorenin comprises three major molecular components that undergo post-translational modifications. Because such endogenous structural changes prevent their detection by most commercial antibodies, we generated polyclonal antibodies against the prorenin prosegment sequence and established a plasma prorenin ELISA. This study was designed to analyze the molecular components of human serum prorenin and to optimize the ELISA for measuring human serum prorenin levels.

Methods: We performed polyacrylamide gel electrophoresis and subsequent immunoblotting to evaluate the molecular forms of human serum prorenin. We established the ELISA protocol to determine the serum prorenin level and compared the serum and plasma prorenin concentrations in 40 healthy volunteers.

Results: Human serum immunoreactive prorenin comprised two major components: a post-translationally modified full-length protein and a smaller protein truncated at the C-terminal renin/prorenin common sequence. An albumin-bound prorenin with higher molecular weight detectable in plasma was absent in human serum. Serum and plasma prorenin levels correlated with each other, whereas serum prorenin levels were lower than plasma prorenin levels. Serum prorenin values were unaffected by any testing postural changes.

Conclusions: Human serum was free from albumin-bound prorenin detectable in plasma and showed reduced prorenin levels. Serum immunoreactive prorenin comprises free prorenin, and stable measurements could be obtained.

Key words: prorenin, serum, post-translational modification, prosegment, ELISA

Introduction

The renin-angiotensin-aldosterone system is one of the most important hormonal mechanisms that regulate blood pressure and maintain fluid and sodium homeostasis. It is currently evaluated clinically by determining the plasma renin activity (PRA) and plasma aldosterone concentrations. However, PRA is an unstable index and fluctuates greatly because of a variety of physiological factors, including postural changes, fluid intake and the effects of drugs. Prorenin, originally discovered as an inactive renin precursor from human amniotic fluid,[1] was found to be present in the blood and kidneys and later in multiple extrarenal organs, such as the brain, adrenal glands, ovaries, placenta, and testis.[2] Prorenin is abundantly produced from the juxtaglomerular cells of the kidney, processed in part to renin, and secreted into the peripheral blood.[3] Its blood concentration has been estimated to be approximately 10 times higher than that of renin.[4] However, plasma prorenin concentrations reported thus far using commercially available enzyme-linked immunosorbent assay (ELISA) kits ranged widely from 60 to 8,000 pg/ml.[5-8] Previously, plasma prorenin levels were extrapolated indirectly by subtracting active renin activity from the total renin activity.[9,10] Alternatively, prorenin levels were estimated by subtracting the active renin concentration from the total renin levels.[11-13]

Prorenin molecules are known to undergo post-translational modifications upon their biosynthesis, such as multiple glycosylations, phosphomannosylation, and formation of disulfide bonds.[14-18] Recently, we found that human plasma prorenin comprises three major molecular components that may be bound to albumin, post-translationally modified, or C-terminally truncated.[19]

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Such structural changes prevent their detection by many conventional antibodies designed to strictly recognize the recombinant prorenin protein. Based on this information, we established a prorenin ELISA using two antibodies against the N-terminal and C-terminal prorenin prosegment sequences suitable for measurement of human plasma prorenin. Surprisingly, plasma prorenin levels determined in healthy subjects were 1,000－10,000-times higher than levels previously reported using commercial ELISA.[19] The present study was designed to analyze the molecular components of human serum prorenin, optimize ELISA for measuring human serum prorenin levels, and determine whether or not serum prorenin levels can be stably determined in clinical laboratories.

Patients and Methods

Plasma and serum sample collection

We enrolled 40 healthy adult volunteers (25 men and 15 women) aged 21－72 (42.1 ± 13.6) years who gave written informed consent. We interviewed the participants to obtain clinical information, including medical history, and participants undergoing current medical treatment were excluded from the analyses. Blood samples were collected in two separate vacuum blood collection tubes containing EDTA-2Na (ethylenediaminetetraacetic acid disodium dihydrate) or a serum separator (Venoject® II vacuum blood collection tube; Terumo, Tokyo). The former was immediately ice-cooled, and the plasma was refrigerated and centrifuged at 3,000 rpm (1,469 × g) for 20 minutes. The latter was coagulated at room temperature for approximately 1 hour, and the serum was then centrifuged. The separated plasma and serum were stored at -30℃ until measurement. The protocol was approved by the Kitasato University Medical School/Hospital Ethics Committee (B19-084), and the study was performed in accordance with the institutional guidelines and regulations.

Materials

Recombinant human prorenin was purchased from Cayman Chemical (Ann Arbor, MI, USA), monoclonal antibodies against human prorenin (clone 4B5-E3) and human renin/prorenin (clone 7D3-E3) from Molecular Innovations (Novi, MI, USA), and goat anti-mouse IgG (H + L) horseradish peroxidase (HRP) conjugate from Bio-Rad Laboratories (Hercules, CA, USA). Polyclonal anti-prorenin prosegment[1－11] IgG was raised in rabbits as described previously[19] and purified using Melon™ gel IgG Spin Purification Kit (Thermo Fisher Scientific, Waltham, MA, USA).

Human serum prorenin ELISA

Human serum prorenin concentration was quantified using prorenin prosegment sandwich enzyme-linked immunosorbent assay (ELISA) essentially as described[19] for detection of plasma prorenin with modifications. Anti-prorenin prosegment [1－11] antibody (0.4 μg/ml) was immobilized onto a 96-well flat-bottomed black microplate at 100 μl/well and allowed to stand at 4℃ for 2 nights. The plate was washed 3 times with 200 μl/well phosphate buffer solution (PBS) containing 0.05% Tween 20 (PBS-T) and blocked with 100 μl/well 1 × casein for 2 hours at room temperature. Enhancer HikariA solution (Nacalai Tesque, Kyoto) was used to serially dilute recombinant human prorenin to 0.1, 0.2, 0.4, 0.6, 0.8, and 1.0 μg/ml and serum samples to 1:20, to which 12,000-fold diluted monoclonal antibody against human prorenin (4B5-E3) was added, mixed and incubated at 37℃ for 105 minutes. The mixture was then applied to anti-prorenin[1－11] IgG-coated 96-well plates prewashed 3 times with PBS-T and further incubated at 37℃ for 105 minutes; after washing the plate 3 times with PBS-T, 100 μl/well goat anti-mouse IgG (H + L) HRP was added at room temperature and incubated for 1 hour in the dark. The plates were rewashed 3 times with PBS-T, overlaid with 100 μl/well 0.5% 3- (4-hydroxyphenyl) propionic acid containing 0.005% H_2O_2 solution, and incubated for 1 hour at room temperature in the dark. The reaction was terminated by adding 100 μl/well 0.1 M glycine-NaOH solution (pH 10.3), and fluorescence was detected using a Spectra Max M2 microplate reader (Molecular Devises, Tokyo) at an excitation wavelength of 320 nm and a fluorescence wavelength of 405 nm. Measurements of all samples were performed in triplicate using 3 wells. The average fluorescence value from triplicate blank wells was subtracted from the average measurement values of each diluted standard- and serum-containing wells to generate the standard plot, which was used to extrapolate the serum concentration.

Immunoblotting

Recombinant human prorenin and thawed human plasma and serum were dissolved in 1 × Laemmli sample buffer (Bio-Rad Laboratories) containing 355 mM 2-mercaptoethanol boiled for 5 minutes and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using 10% Mini-PROTEAN™ TGX™ Precast Protein Gels (Bio-Rad

Laboratories) and Power Pac™ HC Power Supply (Bio-Rad Laboratories). Precision Plus Protein™ Western C™ Protein Standards (Bio-Rad Laboratories) and Precision Plus Protein™ Dual Color Standards (Bio-Rad Laboratories) were used as molecular weight markers. The gels were then transblotted to polyvinylidene difluoride (PVDF) membranes (Immuno-Blot® PVDF Membrane For Protein Blotting; Bio-Rad Laboratories) at 100 V for 1 hour, blocked with 3% bovine serum albumin/Tris-buffered saline for 2 hour at room temperature and then reacted with anti-prorenin antibody diluted with Hikari A solution (Nacalai Tesque) overnight at 4℃. The membrane was then reacted with goat anti-mouse IgG (H+L) HRP conjugate diluted with Hikari B solution (Nacalai Tesque) for 1 hour at room temperature and then visualized with Amersham™ ECL™ Prime Western Blotting Detection Reagents (GE Healthcare Life Sciences; Buckinghamshire, UK) and imaged with ImageQuant LAS4000 (GE Healthcare).

Statistical analyses

Data are expressed as the mean ± standard deviation, with $P < 0.05$ being statistically significant. Serum prorenin values following postural changes were analyzed by analysis of variance. Mann-Whitney U test was used to compare serum prorenin values between the two groups. The linear regression model was used to evaluate the correlation between plasma prorenin and serum prorenin concentrations. Statistical analyses were performed using JMP ver. 5.0.1a (SAS, Cary, NC, USA) and/or GraphPad Prism 5.02 software (GraphPad Software, San Diego, CA, USA).

Results

To compare the molecular sizes of prorenin molecules present in human plasma and serum, we electrophoresed plasma and serum samples simultaneously obtained from same individuals and immunoblotted them using monoclonal antibodies against prorenin prosegment [30－43] (4B5-E3) and renin/prorenin common sequence (7D3-E3). The plasma samples showed three major bands as reported previously[19]: one band that was close to but slightly larger than the recombinant prorenin protein, a larger band, and a smaller band. In contrast, the serum samples did not generate the largest band but generated two bands irrespective of the antibody used (Figure 1). The largest band present in human plasma represented albumin-bound prorenin as described previously and was considered to have been removed during the process of serum separation after clotting and centrifuging the blood samples.

Because serum can be more easily obtained and processed for laboratory tests in daily clinical practice, we optimized the prorenin ELISA for measuring human plasma prorenin in human serum samples. Serially diluted recombinant prorenin protein was used to generate the standard plot of high linearity for 0.1－1 μg/ml recombinant prorenin ($y = 102.45 x - 1.1747$, $R^2 = 0.9954$) (Figure 2). The modified ELISA recognized the serially diluted serum samples in a parallel manner; dilution series of 1:10, 1:15, 1:20, 1:25, and 1:30 from 6 healthy subjects showed high linearity ($R^2 = 0.9992$) and parallelism with the standard plot. The intra- and inter-assay coefficients of variation of the serum samples were 2.85% (n = 7) and 9.97% (n = 5), respectively. The results confirmed the

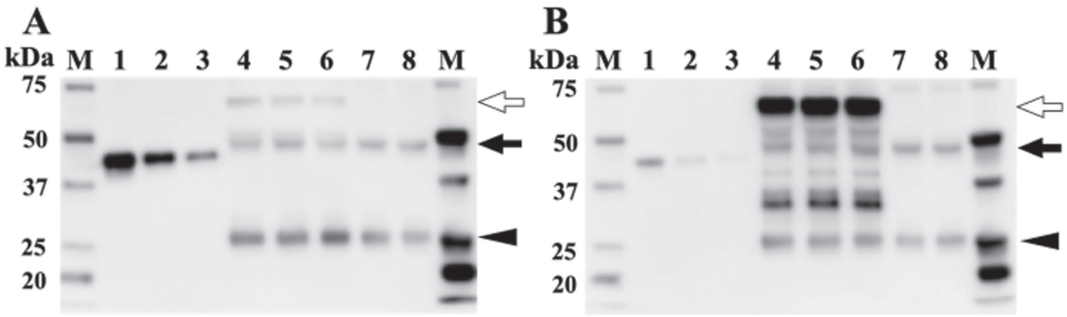

Figure 1. Molecular components of plasma and serum immunoreactive prorenin

Serially diluted recombinant prorenin (2.5, 1.25, and 0.625 ng; lanes 1－3) and human plasma (0.2 μl, lanes 4－6) and serum (0.2 μl, lanes 7, 8) separated by centrifugation immediately (lane 4), 30 minutes (lanes 5, 7), and 60 minutes (lanes 6, 8) after blood withdrawal samples were run on SDS-PAGE and blotted with (**A**) anti-prorenin prosegment [30－43] antibody (4B5-E3, 1:3,000 dilution) or (**B**) anti-renin/prorenin antibody (7D3-E3, 1:5,000 dilution). Immunoreactive prorenins with molecular weight larger (white arrows) and smaller (arrowheads) than that of the full-length prorenin (black arrows) are indicated.

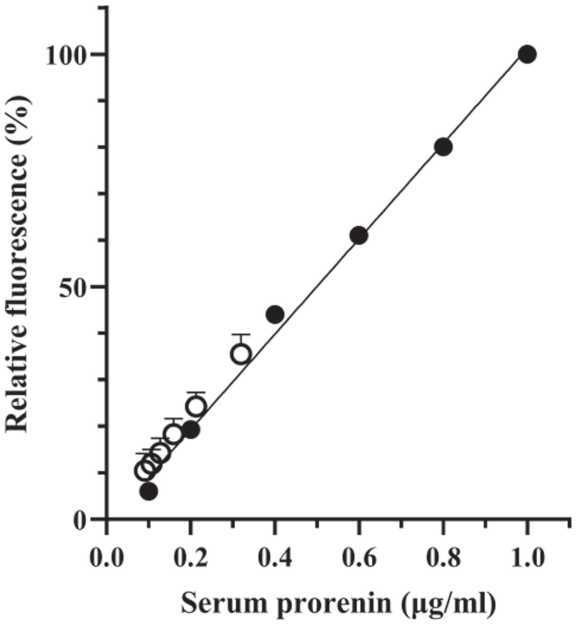

Figure 2. Standard regression plot of a sandwich ELISA using recombinant human prorenin

Human prorenin standard regression plots generated by 9 experiments using serially diluted recombinant prorenin (closed circles and solid line, y = 102.45 x - 1.1747, R^2 = 0.9954) and human serum samples serially diluted to 1:10, 1:15, 1:20, 1:25, and 1:30 (open circles, n = 6) were assessed for their parallelism. The vertical axis represents relative absorbance compared with 1.0 μg/ml standard recombinant prorenin. The data points with bars represent the mean absorbance ± standard deviation (SD).

Table 1. Clinical characteristics of healthy volunteers

Characteristic	Healthy volunteers
Number (male/female)	40 (25/15)
Age (years)	42.1 ± 13.6
Height (cm)	167.1 ± 7.4
Body weight (kg)	61.0 ± 9.3
sBP (mmHg)	115.9 ± 11.8
dBP (mmHg)	70.8 ± 10.4
Pulse rate (bpm)	70.2 ± 11.3
Hemoglobin (g/dl)	14.8 ± 1.4
Albumin (g/dl)	4.5 ± 0.16
Creatinine (mg/dl)	0.78 ± 0.1
HbA1c (%)	5.3 ± 0.3
Triglyceride (mg/dl)	84.7 ± 44.3

sBP, systolic blood pressure; dBP, diastolic blood pressure; HbA1c, glycated hemoglobin

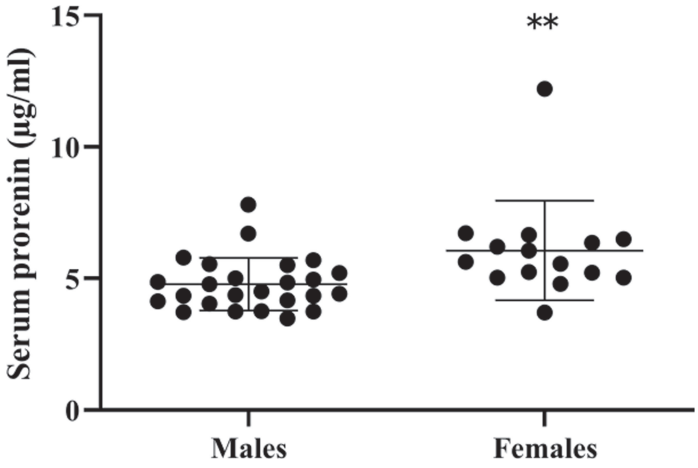

Figure 3. Serum prorenin concentrations in healthy subjects

Human serum prorenin levels were measured in 40 healthy volunteers (25 males, 15 females) using a sandwich ELISA plotting the mean values of triplicate measurements. Each point represents the prorenin concentration determined in a single subject. Horizontal bars indicate the mean absorbance ± standard deviation (SD) of the respective values. **P < 0.01 vs. male subjects, Mann-Whitney U test

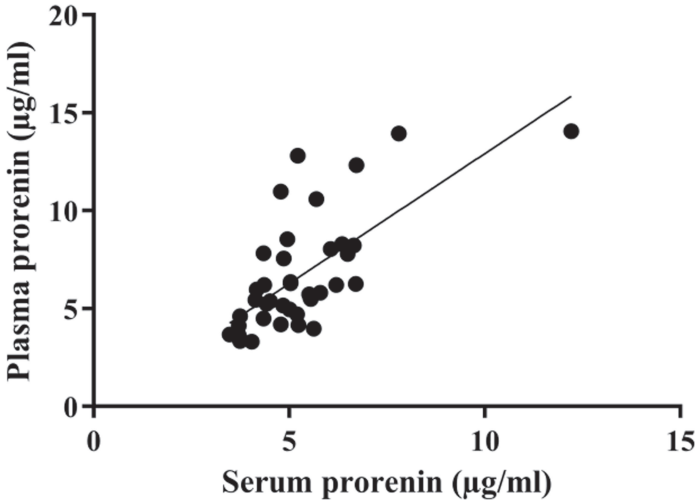

Figure 4. Scatter plots comparing plasma and serum prorenin concentrations

Plasma and serum prorenin levels were measured by respective a sandwich ELISA for paired samples simultaneously withdrawn from 40 healthy volunteers, centrifuged, and separated. The solid line indicates linear regression, r = 0.6909.

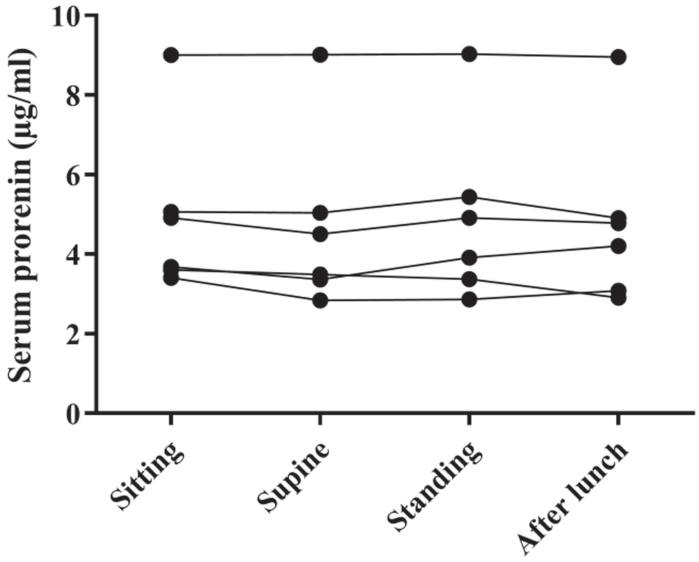

Figure 5. Effects of postural changes on serum prorenin levels

Serum samples were obtained, following an overnight fast, from 6 healthy individuals in the morning after 15 minutes in a sitting position, 30 minutes in a supine position, 60 minutes in a standing position, then in an ambient condition after lunch. Serum prorenin concentrations were determined using the serum prorenin sandwich ELISA.

suitability of the sandwich ELISA for quantifying serum prorenin levels.

The serum prorenin concentration of 40 healthy subjects was 5.3 ± 1.5 μg/ml. The women (6.1 ± 1.9 μg/ml) showed significantly higher values than did the men (4.8 ± 1.0 μg/ml; P = 0.0028; Figure 3). The clinical characteristics of the 40 healthy volunteers are summarized in Table 1. The serum prorenin values were compared with the plasma values using samples simultaneously obtained from the same 40 individuals. The plasma prorenin levels were higher than the serum prorenin levels (6.6 ± 2.9 μg/ml and 5.3 ± 1.5 μg/ml, respectively, $P < 0.05$) and showed significant correlation with the serum prorenin levels ($r = 0.6909$) (Figure 4).

To examine the effect of postural changes and diurnal fluctuations, after fasting overnight, early in the morning, we measured the serum prorenin concentrations in 6 healthy subjects after they sat still for 15 minutes, 30 minutes after recumbency, 60 minutes after standing, and then again after lunch, when they were allowed to move about freely in ambient conditions, at 2:00 p.m. Serum prorenin concentrations did not change significantly after any variations in the testing positions at any of the 4 time points (Figure 5).

Discussion

We have recently identified three distinct molecular forms of prorenin in human plasma: an albumin-bound form, post-translationally modified full-length prorenin, and a C-terminally truncated fragment.[19] Furthermore, using 2 antibodies against the N-terminal and C-terminal human prorenin prosegment sequences, we established a prorenin sandwich ELISA and determined the human plasma prorenin concentration. Remarkably, the directly measured plasma prorenin concentration was 1,000－10,000-times higher than values previously reported with conventional commercially available ELISA kits. We confirmed the ability of the prorenin prosegment antibody to capture full-length prorenin molecules and performed semiquantitative immunoblotting analysis using several prorenin antibodies.[19] The results supported the approximate human plasma prorenin concentrations determined in this study. In the present study, we investigated the molecular forms of serum prorenin and the blood concentration measurement system to determine if serum prorenin concentration measurement can also be used as a clinical laboratory test.

We first compared the molecular sizes of immunoreactive plasma and serum prorenin using anti-prorenin prosegment [30－43] antibody (4B5-E3) and anti-renin/prorenin antibody (7D3-E3). Western blot analysis revealed that the band with the largest molecular weight detected in plasma samples was not detected in human serum samples obtained simultaneously. This band was confirmed to comprise prorenin and albumin

and disappeared when the plasma was pretreated with an albumin removal column.[19] Gel permeation chromatography of a mixture of recombinant prorenin and purified albumin protein generated a larger-molecular-weight immunoreactive prorenin fraction that coincided with plasma immunoreactive prorenin.[19] These results indicated that this immunoreactive prorenin component is an endogenous prorenin bound to albumin. The disappearance of this component in serum suggests that the albumin-bound prorenin was removed during the serum separation process.

We optimized the plasma prorenin ELISA method to measure serum prorenin levels because it was revealed that serum comprises only two components, post-transcriptionally modified full-length prorenin and its C-terminally cleaved peptide, and did not contain the albumin-bound prorenin. ELISA for human serum prorenin generated a standard regression plot showing high linearity and parallelism with serially diluted serum samples. The assay detection limit, as well as intra- and inter-assay coefficients of variation, was sufficiently low. Thus, this sandwich ELISA is considered to be suitable for determining serum prorenin levels.

We measured the serum and plasma prorenin concentrations in 40 healthy subjects and compared the levels using samples obtained simultaneously from those same subjects. A significant correlation was observed between the plasma and serum prorenin concentrations, and the plasma prorenin concentrations were approximately 1.2-times higher than were the serum prorenin concentrations. The lower prorenin levels in serum samples than in the plasma samples were considered to be because of the removal of albumin-bound prorenin in the serum samples. The ratio of plasma prorenin concentration to serum prorenin concentration varied in each individual. Some subjects showed relatively higher plasma prorenin concentrations than serum prorenin concentrations. This suggests that the amount of prorenin molecules bound to albumin varies in each individual.

The results also showed that the serum prorenin level in females was significantly higher than that in males. PRA is known to increase during the luteal phase of the menstrual cycle,[20] whereas progesterone administration in males augments PRA.[21] Our healthy subjects comprised females in their reproductive age, which may have contributed to the increased serum levels. Therefore, it is speculated that progesterone enhances the release of prorenin molecules into the systemic circulation, thereby stimulating PRA.

We examined the effects of postural changes on serum prorenin concentrations and found that the levels were not significantly affected by any of the variations in the testing conditions. PRA is the only available clinical biomarker representing renin/prorenin and an excellent biomarker for evaluating intravascular fluid status. PRA is significantly affected by the blood collection posture, acute changes in extracellular fluid volume status, and/or medication and requires maintaining a recumbent position and preferably overnight fasting before collecting blood samples. PRA does not reflect steady-state production/release of renin/prorenin molecules. The current results showed that the serum prorenin concentration is very stable and can be measured using serum samples obtained at ambient conditions. Further studies are warranted to establish the overall usefulness of serum prorenin levels in the clinical diagnoses of cardiovascular and renal diseases.

In conclusion, these findings established a sandwich ELISA method for measuring human serum prorenin. Human serum does not contain albumin-bound prorenin but showed lower prorenin levels than those in plasma. However, both human serum and plasma prorenin levels correlate significantly and provide stable results.

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Conflicts of Interest: None

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