Hypertension

RESEARCH LETTER

Concerns on the Specificity of Commercial ELISAs for the Measurement of Angiotensin (1–7) and Angiotensin II in Human Plasma

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The current coronavirus disease 2019 (COVID-19) pandemic and the interaction of severe acute respiratory syndrome coronavirus 2 virus with the renin-angiotensin system to bind and internalize ACE2 (angiotensin-converting enzyme 2, EC 3.4.17.23) has underscored the need for accurate assessment of the renin-angiotensin system in severe acute respiratory syndrome coronavirus 2-infected patients, particularly those with underlying cardiovascular disease including hypertension, heart failure, and kidney disease.[1,2] Liu et al[3] initially reported that patients with COVID-19 had significantly elevated plasma levels of Ang II (angiotensin II) as compared to healthy patients and that plasma Ang II levels correlated with a greater viral load and reduced lung function in the patients with COVID-19.[3] This study used a sensitive ELISA to directly quantify Ang II in plasma that requires a small sample volume (50 μL) and obviates the need for prior sample extraction or enrichment. These commercial ELISAs have frequently been used to quantify Ang II and Ang (1–7) (angiotensin (1–7)) directly in plasma and serum; however, the reported peptide levels vary widely in these studies which raises concern on the specificity of the 2 assays.[4] Moreover, the recommended sample size for these assays appears too low to quantify Ang II (50 uL) or Ang (1-7) (10 uL) in plasma. To address this potential concern, the present study compared Ang (1–7) and Ang II content in human plasma as assessed by these 2 commercial ELISAs to that obtained from validated radioimmunoassays used in our laboratory.[5,6]

Plasmas from deidentified patient samples with elevated plasma renin activities (>12 ng Ang I/mL/h) to ensure measurable peptide values for Ang (1–7) and Ang II were thawed on ice and pooled for comparison of peptide values between the ELISA and radioimmunoassay methods. Blood samples were collected directly into EDTA-containing tubes with no additional peptidase inhibitors to obviate any interference with the direct plasma ELISA measurements. For the ELISAs, the pooled plasma samples (10 μL for Ang (1-7) or 50 μL for Ang II per the exact ELISA instructions) were diluted in each ELISA buffer to 100 μL and directly applied to the antibody-coated plate. Aliquots (1 mL) of the plasma pool were also extracted on SepPak C18 columns (200 mg, Millipore-Sigma, Burlington, MA) activated with 80% methanol (5 mL) and 0.1% trifluoroacetic acid (5 mL) in MilliQ water. The plasma was initially diluted with 0.1% trifluoroacetic acid (1 mL) and applied to the SepPak columns at room temperature, which was subsequently washed with 0.1% trifluoroacetic acid (5 mL), MilliQ water (5 mL), and the peptides eluted in 80% methanol (1 mL). The eluate was evaporated in a vacuum centrifuge and reconstituted in either the ELISA or radioimmunoassay buffers. Ang (1–7) and Ang II ELISA standards were added to the plasma to determine peptide recovery from SepPak extraction, as well as for the direct plasma measurements. In addition, our Ang (1–7) and Ang II standards (Bachem, Torrance, CA) validated by high performance liquid chromatography and radioimmunoassay were evaluated for each ELISA following dilution in the ELISA buffer. Cross-reactivity of human angiotensinogen (Aogen, 20 μg/mL, Abcam, Cambridge, MA) for both ELISAs was also determined given the abundant content of Aogen in plasma and serum (≈20–80 μg/mL).[5] Peptide content was expressed as picograms per mL

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Nonstandard Abbreviations and Acronyms

ACE2	angiotensin-converting enzyme 2
Ang (1–7)	angiotensin (1–7)
Ang II	angiotensin II
COVID-19	coronavirus disease 2019

(pg/mL). The human Ang (1–7) and Ang II ELISA kits were obtained from Cloud Clone Corporation (formerly USCN Life Science and Technology, Missouri City, TX), the human Ang II radioimmunoassay was obtained from IBL America (Minneapolis, MN, sensitivity of 2.0 pg/mL) and the Ang (1–7) radioimmunoassay (sensitivity of 2.5 pg/mL) was developed in our laboratory.[5] Data are the mean±SEM (N=3 determinations or extractions per sample) and analyzed by 1-way ANOVA with Tukey post hoc analysis, $P<0.05$ for statistical significance.

As shown in Figure [A], the direct plasma Ang (1–7) value by ELISA was 779±71 pg/mL. Following SepPak extraction of plasma, Ang (1–7) levels by ELISA were below the assay sensitivity (<7 pg/mL, but extrapolated to 3.0±0.1 pg/mL). Addition of the ELISA Ang (1–7) standard (50 pg/mL) to plasma for direct measurement increased the plasma content to 938±43 pg/mL with a peptide recovery of 113%. Addition of the ELISA Ang (1–7) standard (50 pg/mL) to the plasma sample before extraction increased content to 41±4 pg/mL with a peptide recovery of 82%. The Ang (1–7) ELISA failed to directly detect the Bachem Ang (1–7) standard over a concentration range of 100 to 20 000 pg/mL (data not shown). The Ang (1–7) value in extracted plasma by radioimmunoassay was 46±5 pg/mL (Figure [A]).

As shown in Figure [B], the direct Ang II value in pooled plasma by ELISA was 214±9 pg/mL. Following SepPak extraction, Ang II content fell below the assay sensitivity (<9 pg/mL, but extrapolated to 4±1 pg/mL). Addition of the ELISA Ang II standard (50 pg/mL) to plasma for direct measurement increased Ang II levels to 617±77 pg/mL with a peptide recovery of 234%. Addition of the ELISA Ang II standard (500 pg/mL) to plasma before extraction increased content to 513±52 pg/mL with a peptide recovery of 103%. The Ang II ELISA also failed to detect the Bachem Ang II standard over a range of 100 to 20 000 pg/mL (data not shown). The Ang II radioimmunoassay value in extracted plasma was 138±4 pg/mL (Figure [B]). Finally, both ELISAs did not recognize the human Aogen standard at a concentration of 20 μg/mL (data not shown).

Studies using the 2 commercial ELISAs have reported a wide range of Ang (1–7) and Ang II values in plasma and serum varying from 2 to 3500 pg/mL.[3,4] An apparent advantage of these ELISAs over other assays is the direct measurement of plasma or serum samples which simplifies the assay procedure and obviates potential loss

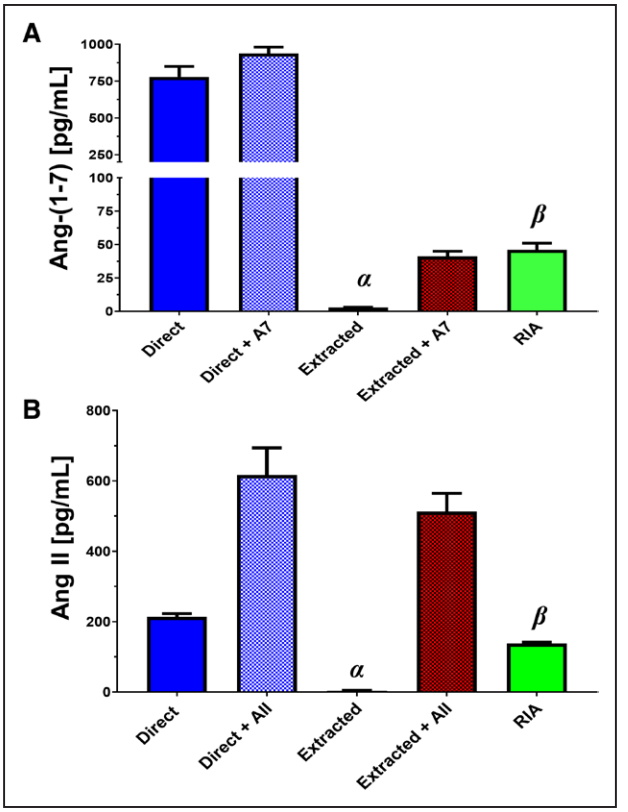

Figure. Comparison of Ang (1–7) (angiotensin (1–7)) and Ang II (angiotensin II) peptide levels in pooled human plasma by ELISA and radioimmunoassay (RIA) methods.
A, Ang (1–7) content in plasma (direct) or extracted plasma (extracted) by ELISA or in 1 mL extracted plasma by RIA. Direct plasma measurement with 50 pg/mL of ELISA Ang (1–7) added (direct + A7) or extracted plasma with 50 pg/mL of ELISA Ang (1–7) added (extracted + A7) by ELISA. **B**, Ang II content in plasma (direct) and extracted plasma (extracted) by ELISA or extracted plasma by RIA. Direct plasma with 50 pg/mL of ELISA Ang II added (direct + AII) or extracted plasma with 500 pg/mL of ELISA Ang II added (extracted + AII) by ELISA. Data are means±SEM, N=3; α $P<0.05$ vs direct or RIA, β $P<0.05$ vs direct.

of the peptide during extraction. However, we obtained strikingly different peptide values between the direct and extracted plasma samples with the 2 ELISAs. These differences do not reflect peptide loss or interference by the plasma extract since recoveries of the ELISA Ang (1–7) and Ang II standards were >80%, nor insufficient sample volume as 1 mL of extracted plasma was assayed versus 0.01 and 0.05 mL of plasma directly for Ang (1–7) and Ang II, respectively. The radioimmunoassay values for Ang (1–7) and Ang II in the plasma extract were 46 and 138 pg/mL, respectively, which are well within the stated detection limits of these ELISAs and in the expected range for Ang (1–7) and Ang II in human plasma from patients with high plasma renin activities. Ang (1–7) and Ang II values from individuals with normal plasma renin activities are more likely in the 5 to 50 pg/mL (≈5–50 pmol/L) range[6] (for a more detailed review of expected angiotensin values). Both ELISAs failed to

detect human Aogen, so it is unlikely that the high values by direct plasma measurement reflect cross-reactivity with Aogen. We speculate that the two ELISAs detect other unidentified substances in plasma which are effectively removed by SepPak extraction. Indeed, solid-phase extraction such as the SepPak C18 column is a key step to quantify angiotensins as this removes contaminating substances, concentrates the peptides in a small volume of solvent that can be readily evaporated to effectively increase detection levels of the assay, and provides some degree of validation of the peptide values, particularly if recoveries are also determined.[6] As to the inability of these ELISAs to recognize the Bachem Ang (1–7) and Ang II standards, either the sensitivities of the ELISAs are far lower (worse) than stated by the manufacturer or these assays do not detect authentic Ang (1–7) and Ang II. This may also explain the failure of both ELISAs to detect Ang (1–7) and Ang II in the extracted plasma samples. Nonetheless, we cannot recommend that these assays provide an accurate assessment of Ang (1–7) or Ang II in direct or extracted plasma samples.

The quantification of Ang (1–7) and Ang II is challenging as endogenous levels of these peptides are quite low ranging from 10^{-12} to 10^{-10} grams per mL plasma or per gram tissue.[6] For over 40 years, radioimmunoassays were the standard method to measure angiotensins given their sensitivity and relative specificity. ELISAs have supplanted radioimmunoassays as these immunoassays do not require radioactive ligands with limited half-lives, antibody precipitation steps, or gamma-counters; their main requirement is an inexpensive plate reader for signal detection. More recently, mass spectroscopy combined with ultrahigh-pressure liquid chromatography has been applied for angiotensin quantification that obviates antibody-based detection altogether; however, few laboratories possess the specialized equipment and expertise for this analytical approach, particularly for a high throughput analysis of samples.[6,7] The advent of COVID-19 and the viral interaction of severe acute respiratory syndrome coronavirus 2 with ACE2 requires an accurate assessment of Ang II and Ang (1–7) in patients.[1] Although costs prohibited comparisons with additional commercial assays for Ang II and Ang (1–7) or other angiotensins (Ang I, Ang (1–12), Ang (1–9)], we recommend several precautions for investigators that quantify endogenous angiotensins with ELISAs or radioimmunoassays: (1) the assessment of plasma rather than serum in which the blood is collected into peptidase inhibitors, rapidly processed to obtain the plasma, and stored at −80 °C or immediately extracted; (2) serum is not recommended as an alternative to plasma as serum is allowed to clot for extended periods without the presence of peptidase inhibitors that may lead to artifactual generation of high peptide levels that do not reflect endogenous content; (3) an adequate volume of sample and enrichment step which yields peptide values that are well within the detection limit of the assay; (4) solid-phase extraction of all biological samples rather than direct assay or precipitation with organic solvents; (5) assessment of peptide recovery for the extraction procedure; (6) validation of the assay with known angiotensin standards distinct from those provided in the commercial kit; and (7) validation of the immunoreactive content of the sample or sample pool if possible, particularly if high peptide values outside the expected range are detected by the assay.[6]

ARTICLE INFORMATION

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Disclosures

None.

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