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Plasma Renin Activity by Liquid Chromatography–Tandem Mass Spectrometry (LC-MS/MS): Development of a Prototypical Clinical Assay Reveals a Subpopulation of Human Plasma Samples with Substantial Peptidase Activity

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BACKGROUND: For management and treatment of secondary hypertension, plasma renin activity (PRA) assay is considered an essential diagnostic tool. We developed a liquid chromatography–tandem mass spectrometry (LC-MS/MS)-based approach to PRA offering improvements in laboratory workflow and throughput. During development, we observed a substantial number of clinical samples that have strong degradation activity toward angiotensin (Ang) I during generation. A preliminary characterization of this degradation activity was performed, and we provide here a method by which this degradation can be monitored via the addition of an isotope-labeled degradation standard.

METHODS: Automated online sample extraction coupled with HPLC was used to isolate Ang I and internal standard from plasma. The effluent from the analytical column was directed to a triple quadruple MS operated in selected reaction monitoring mode, monitoring the a_5 and b_5 product ions from the $[M+3H]^{+3}$ precursors. Routine analysis could be achieved with as little as 150 μL plasma.

RESULTS: We identified both C-terminal and N-terminal degradation products of Ang I using isotope-labeled peptides as controls and substrates. In 2%–5% of patient samples, the degradation essentially eliminated any Ang I produced during generation.

CONCLUSIONS: Our method requires reduced sample handling when compared with an RIA and eliminates the need for extended generation times for samples with low renin activity. Degradation of Ang I during generation appears to be a confounding variable in the interpretation of results from some clinical samples. Samples with profound degradation activity can be identified using a degradation standard that is added at the start of generation.

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In the treatment of hypertension, plasma renin activity (PRA)[1] assay is used to assess the capacity of circulating renin and angiotensinogen to generate the pressor peptide angiotensin (Ang) II via quantitative analysis of the upstream peptide precursor Ang I. Although Ang II is recognized as a key active component in the regulation of blood pressure in humans, accurate measurement of the circulating concentration of Ang II is considered to be technically challenging because of instability of Ang II in blood samples and high cross-reactivity of anti–Ang II antibodies to degradation products *(1–3)*. The measurement of Ang I as a surrogate for the capacity of the renin system to generate Ang II was reported by Sealey and Laragh in 1975 *(4)*, and a similar method was outlined by Fyhrquist et al. in 1976 *(5)*. This RIA method has a history of being robust and reliable when appropriate attention is paid to assay conditions *(6)*.

The RIA requires long assay times and laborious sample handling. The primary difficulties arise from the limited dynamic range of the RIA, which requires dilution and/or reincubation of samples with high PRA or extended incubation for samples where the PRA is low. In our lab, 30%–40% of samples require additional effort beyond the first assay to report a result due to these constraints. To address these limitations of the RIA, we developed an isotope dilution liquid chromatography–tandem mass spectrometry (LC-MS/MS) method for the quantitative determination of Ang I in human plasma. Although an LC-MS/MS assay for Ang

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[1] Nonstandard abbreviations: PRA, plasma renin activity; Ang, angiotensin; LC-MS/MS, liquid chromatography–tandem mass spectrometry; AEBSF, aminoethyl benzenesulfonyl fluoride; IS, internal standard; DS, degradation standard; SRM, selected reaction monitoring; QC, quality control; ARR, aldosterone-renin ratio.

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I was previously reported by Fredline et al. *(7)*, we believe that our assay offers improvements in assay performance and workflow. In addition, we do not observe significantly lower results by LC-MS/MS when compared with the RIA *(7)*.

During assay development, we observed degradation of Ang I in patient samples that required extended incubation times. For most samples, the degree of degradation is mild and the impact is ameliorated by short generation times. However, some samples presented a striking degree of degradation, and our assay enabled these samples to be easily identified. While the proteolytic activity of human plasma and serum has long been appreciated, intense proteomic discovery efforts have started to illuminate the dynamic nature of plasma and the attendant concerns *(8–10)*.

Materials and Methods

HPLC grade acetonitrile and water were purchased from Burdick and Jackson. High purity formic acid, maleic anhydride, BSA, aminoethyl benzenesulfonyl fluoride (AEBSF), and synthetic angiotensin I were obtained from Fluka. Isotope-labeled peptides; the analytical internal standard (IS) DR[V^{13}C,^{15}N]YIHPFHL; the degradation standard (DS) DR[V^{13}C,^{15}N]Y[I^{13}C,^{15}N]HPFHL; and the DS DRVYIH[P^{13}C,^{15}N]FHL and DRVYI[H^{15}N] PF[H^{15}N]L used for precursor ion and targeted selected reaction monitoring (SRM) studies were purchased from Sigma Life Sciences or Cambridge Isotope Laboratories. Selected synthetic peptides for degradation studies were purchased from Anaspec, Sigma, and Austral Bio (peptide 1, EGVNDNEEGFFSAR; peptide 2, LSGVQVQQHSQAL; peptide 3, pyrELYENK-PRRPYIL; peptide 4, GFYFNKPTGYGSASR; peptide 5, YKEGYNVYG).

PREPARATION OF INTERNAL STANDARDS, CALIBRATORS, AND CONTROLS

Because measurable amounts of Ang I were detected in commercially available stripped serum products, calibrators were made up in an artificial serum matrix of PBS (0.01 mol/L phosphate, 2.7 mmol/L KCl, 137 mmol/L NaCl, pH 7.4) containing 1 mmol/L AEBSF and supplemented with 45 g/L BSA. A working stock solution of Ang I was prepared by dilution into an artificial matrix and further diluted in artificial serum to generate a series of calibrators from 0 to 140.6 μg/L. Aliquots of calibrator solution were stored at −80 °C until use. The IS and DS were supplied as prequantified aliquots of lyophilized peptide. Spiked pools for use as quality control (QC) samples were prepared by dilution of angiotensin I stock in artificial serum to provide three pools at 1.1, 8.7, and 35 μg/L. Bio-Rad Lyphocheck controls were prepared as indicated in the kit insert, and ranges for each lot were determined using the LC-MS/MS protocol presented here.

PATIENT SAMPLES

Nonhypertensive control participants provided written informed consent before blood draw. An institutional review board waiver was obtained for collection of samples from nonhypertensive control participants and for utilization of residual deidentified clinical samples. In all cases, blood was collected into potassium EDTA tubes, immediately processed to obtain plasma, and kept frozen at −20 °C until analysis. All samples for method comparison studies were processed according to the Sealey protocol for the RIA *(4)* and by the method described here for analysis by LC-MS/MS.

SAMPLE PREPARATION

A 250-μL aliquot of EDTA plasma or Bio-Rad control was mixed with 25 μL generation cocktail (0.275 mol/L maleic acid, pH 1.2, 5 mmol/L AEBSF, 45 nmol/L DS). The DS was added at the start of generation, and its recovery was determined during assay by LC-MS/MS. The samples were agitated and then incubated at 37 °C for 3 h. After incubation, 275 μL of 10% formic acid containing 8 nmol/mL of IS was added to stop the reaction. Calibrators were likewise acidified. The acidified samples were filtered before mass spectrometry. For degradation studies, synthetic Ang I with isotope-labeled proline or histidine was used as a substrate for the degradation activity and added to plasma at 1500 nmol/L. Incubation times were adjusted for selected degradation experiments.

AUTOMATED PREPARATIVE AND ANALYTICAL CHROMATOGRAPHY

Chromatographic separation of Ang I from matrix components before tandem MS was accomplished with an Aria TX-4 (Thermo-Fisher), a fully automated online chromatography system for sample preparation and separation. Generated Ang I, DS, and IS were extracted from 100 μL acidified human plasma using an HLB cartridge column (20 × 2.1 mm, 25 μmol/L particle size) (Waters). After injection of the acidified plasma, the extraction cartridge was washed with 95% solvent A (0.1% formic acid in water). The analytes were then back-flushed off the extraction cartridge onto the analytical column. Analytical separation of Ang I and related standards was performed with an X-Bridge C18 column (50 × 2.1, 5 μm particle size) (Waters) using a gradient of 5%–30% solvent B (acetonitrile + 0.1% formic acid) in solvent A over 2.5 min. The column was briefly washed at 80% B and reequilibrated at 5% solvent B for 45 s.

TANDEM MASS SPECTROMETRY AND DATA PROCESSING

A Thermo-Fisher TSQ Quantum Ultra triple quadrupole equipped with a heated electrospray ionization probe was used to perform the analysis. Analytes were quantified using SRM in positive ion mode under the following conditions: ionization voltage, 3000 V; sheath gas pressure, 30 arbitrary units; auxiliary gas pressure, 30 arbitrary units; capillary temperature, 250 °C; and vaporizer temperature, 350 °C. Two *m/z* transitions were used for each precursor: Ang I, *m/z* 433–619 and *m/z* 433–641; IS, *m/z* 435–625 and *m/z* 435–653; DS *m/z* 437–638 and *m/z* 437–660. In each instance, the ions used were a_5 and b_5 product ions, respectively. The two transitions for each analyte were summed and used for quantitative analysis. A characteristic ion ratio of 70:30 was monitored for the b_5/a_5 ion pair for each analyte. The analyte peak area to IS peak area ratio was plotted against concentration to obtain calibration curves, which were calculated using weighted (1/x) linear, least squares regression. The apparent fraction degraded was calculated as the patient sample DS/IS ratio divided by the mean nondegrading control DS/IS ratio. Reduction of raw MS peak areas to concentrations was performed using LCQuan 2.5 software (Thermo-Fisher). Results were reported as the rate of Ang I generated in $\mu g \cdot L^{-1} \cdot h^{-1}$ after correction for generation time and dilution.

Precursor ion experiments were carried out using transmission conditions that selected the immonium ions of isotope-labeled proline or histidine in Q3 (*m/z* 75 or 113, respectively), while Q1 was scanned over *m/z* regions that would be associated with N- or C-terminal–directed degradation of the labeled peptide. The HPLC separation of samples for precursor ion experiments was identical to the separation for the assay of Ang I described above. Results from precursor ion scanning experiments were confirmed by performing the analyses a second time using targeted SRM. Additional details regarding mass spectrometry experiments can be found in Sections 1 and 2 of the Data Supplement that accompanies the online version of this article at http://www.clinchem.org/content/vol56/issue10.

Results

ASSAY PERFORMANCE AND COMPARISON

The method comparison data are shown in Fig. 1. Additional details of the method validation are available in Section 3 of the Data Supplement. Because of minor variations in the reference interval (0.24–5.5 μg/L/h) when compared with results obtained via the RIA methodology (0.5–5.0 μg/L/h) and because of the importance of the PRA assay result in determining the aldosterone-renin ratio (ARR) *(11, 12)*, we chose to check the ARR reference interval by assaying samples from nonhypertensive control subjects using the LC-MS/MS PRA assay and LC-MS/MS aldosterone methods. The ARR reference interval under these conditions was established as 0.9–28.9. Our data are in agreement with the upper cutoff value of 30 (PRA in μg/L/h, plasma aldosterone in ng/dL) [Copyeditor/printer: The units of ng/dL are acceptable here, these are the units used in the reference studies] for the ARR cited by the Endocrine Society Clinical Practice Guideline *(13)*.

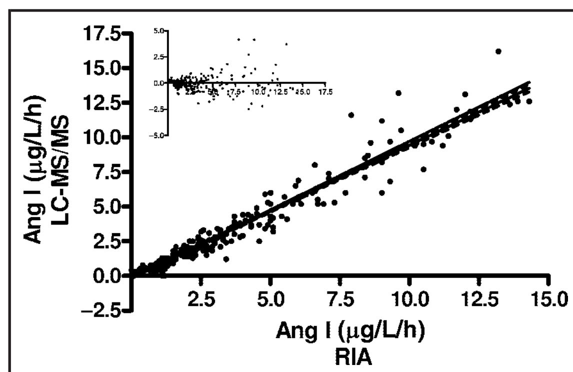

Fig. 1. Method comparison between RIA and LC-MS/MS.

All samples for LC-MS/MS were generated for 3 h. For RIA data, samples with PRA <0.65 μg/L/h were generated for 18 h; samples with PRA >0.65 μg/L/h were generated for 3 h. For samples with PRA >14.9 μg/L/h, the 3 h generated sample was diluted 1:5. The unity line is indicated as a solid line and the Deming regression as a dashed line. Residuals are plotted on the inset.

In the exploratory phase of assay development, we observed a mild but consistent negative bias of the RIA to LC-MS/MS results when the RIA result was <0.65 μg/L/h. For these low PRA samples, the generation had been carried out for 18 h before RIA, but LC-MS/MS samples had been incubated for only 3 h. In addition to this general bias, we also noted several outliers where the 18-h and 3-h results were dramatically different, suggesting Ang I degradation. The possibility of Ang I degradation during generation has been suggested by other authors, with the most recent data reported by Derkx and Schalekamp *(14)*, who observed 20%–59% loss after 18 h. Fredline et al. *(7)* also showed a generation time-dependent bias with a loss of Ang I when PRA was >2.0 μg/L/h.

To investigate the bias between the 3- and 18-h–generated samples in more detail, we obtained a second isotope of Ang I containing two labeled amino acids for use as a DS. By incorporating the DS into the generation cocktail and monitoring its disappearance in plasma, we were able to directly assess the loss of Ang I

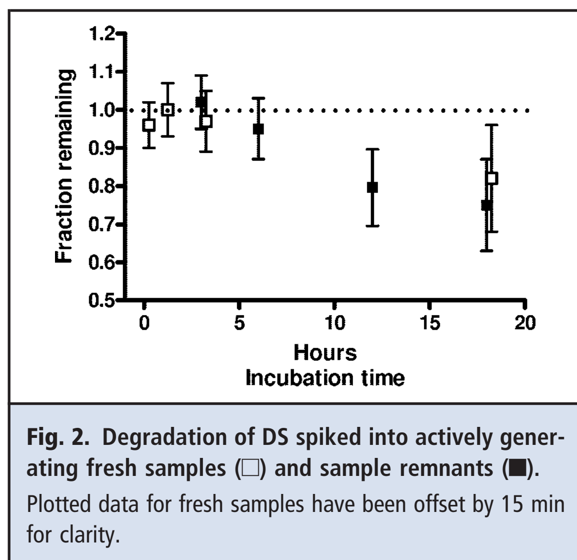

Fig. 2. Degradation of DS spiked into actively generating fresh samples (□) and sample remnants (■). Plotted data for fresh samples have been offset by 15 min for clarity.

as a function of time. To calculate the percent apparent degradation against an authentic nondegrading control, DS was spiked into Bio-Rad Lyphocheck QC pools (stabilized human plasma) or artificial serum matrix. Both of these sample types demonstrated negligible degradation activity over an 18-h incubation. A control DS/IS ratio was determined for each incubation time point by averaging the results from the three Bio-Rad standards and the artificial serum matrix. In parallel, a DS/IS ratio was calculated for each patient sample. The apparent fraction recovered was then established as the patient sample DS/IS ratio divided by the control DS/IS ratio.

Loss of labeled Ang I with time was studied with a set of freshly collected patient samples (n = 128). Aliquots were taken at 0, 1, 3, and 18 h during the generation. This sample set showed a mean loss of 19% labeled Ang I at 18 h with no loss from 1 to 3 h (Fig. 2). In this, as well as another sample set, the observed degree of degradation was highly variable, and this heterogeneity manifested itself by the increasing standard deviation of apparent recovery because the incubation time was extended. No dependence between PRA and degradation activity was noted.

In addition, we identified several samples that showed substantial loss of DS during generation (>50% in 3 h) and some for which the loss of DS was essentially complete. The existence of these samples suggests that there may be a subpopulation of patients where very low PRA measurements result from Ang I loss rather than very low renin activity.

Concerns that this strong degradation activity could be attributed to poor control of experimental conditions *(6, 15, 16)* were eliminated because of our observation that degradation could be replicated, supplementation of the reaction with additional EDTA or replacement of AEBSF with PMSF did not inhibit the degradation process, and pH control was noted during generation (n = 144, pH 5.7 ± 0.2). We also examined whether the process of pH adjustment of plasma with generation buffer containing maleic acid affected the degradation activity. Degradation activity was unchanged after dilution with water, stripped serum, or maleic acid. For actively degrading samples, we determined that degradation activity could be titrated by dilution. In addition, heat or acid treatment of plasma also eliminated the degradation activity. All of these observations suggest an enzymatic activity specific to the patient sample displaying the effect.

DISTRIBUTION OF DEGRADATION WITH RESPECT TO RENIN ACTIVITY VALUES

Given the presence of degradation in patient samples, understanding the frequency of degradation with respect to PRA assay result was an important element in assessing the clinical utility of a PRA assay or ARR result when degradation was present. We collected 371 patient samples and measured the PRA values and degradation by LC-MS/MS. In this set, 8 of 63 (12%) showed a complete loss of DS after 3 h of generation and had PRA values in the range of <0.04–0.15 μg/L/h. However, the overall frequency of these highly degrading samples in the whole sample population was low (approximately 2%).

SPECIFICITY OF DEGRADATION

The specificity of degradation in patient plasma was examined by monitoring the concomitant stability of a cocktail of 5 peptides unrelated to Ang I. The peptides were selected to represent a variety of lengths and compositions. Specific transitions for each peptide were designed and a multiplex SRM experiment was run to follow each peptide along with DS and IS. Aliquots were taken at 0, 15, and 30 min for analysis. As shown in Fig. 3, degradation activity appeared to be broad but not entirely nonspecific. One spiked peptide (peptide 3) appeared to be more stable, while the others showed varying degrees of degradation. In fact, Ang I itself appeared to be mildly resistant to the degradation activity in comparison to some of the other peptides. Patient samples that were not identified as degraders showed no loss of any of the spiked peptides.

CHARACTERIZATION OF DEGRADATION PRODUCTS

To understand the possible mechanisms of degradation, we characterized potential degradation products. Patient samples with high Ang I degradation activity (>80% loss after a 3-h generation) were subjected to a short 30-min generation using Ang I labeled with proline (^{13}C, ^{15}N) or histidine (^{15}N). Using precursor ion scan

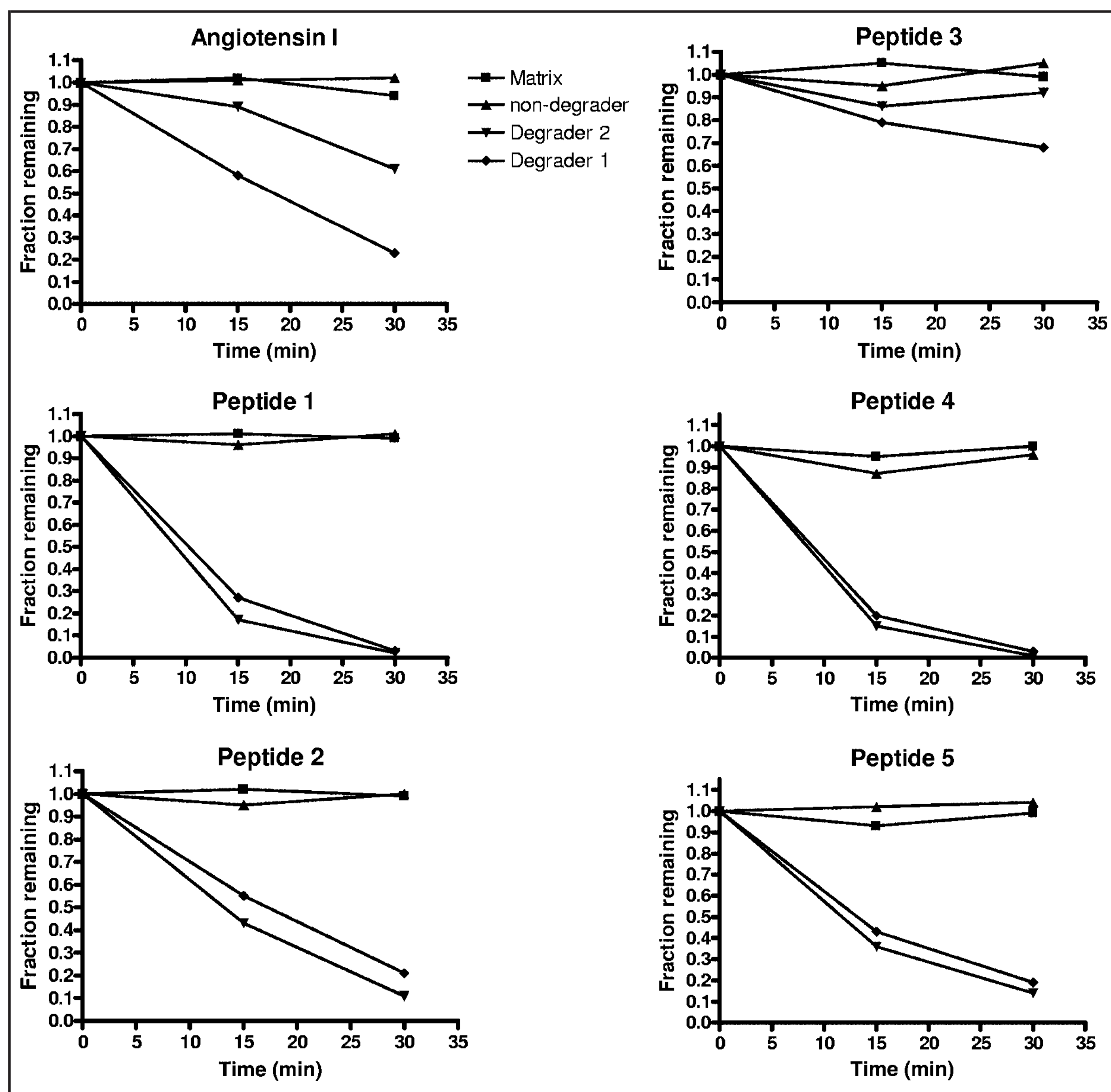

Fig. 3. Stability of spiked, non-angiotensin–related peptides in degrading and nondegrading plasma.
Data for artificial serum (■), nondegrading patient sample (▲), degrading patient sample 1 (▼), and degrading patient sample 2 (◆) are shown.

data to identify degradation candidates with subsequent confirmation by targeted SRM, both N- and C-terminal degradation products were identified (Fig. 4). Nondegrading samples, artificial serum, and Bio-Rad Lyphocheck QC pools demonstrated no losses or changes.

In precursor ion experiments, we were able to take advantage of the appearance of multiple charge states of peptides to assist in the preliminary identification of degradation products. The spectrum in Fig. 5 shows the +2 and +3 charge states for two different angiotensin I degradation products that coeluted during the LC-MS/MS experiment. By manual inspection of data acquired from many samples, various degrees of C-terminal and/or N-terminal degradation were confirmed (Fig. 6).

Discussion

The amount of angiotensinase activity in plasma and its impact on PRA measurement has been shown in

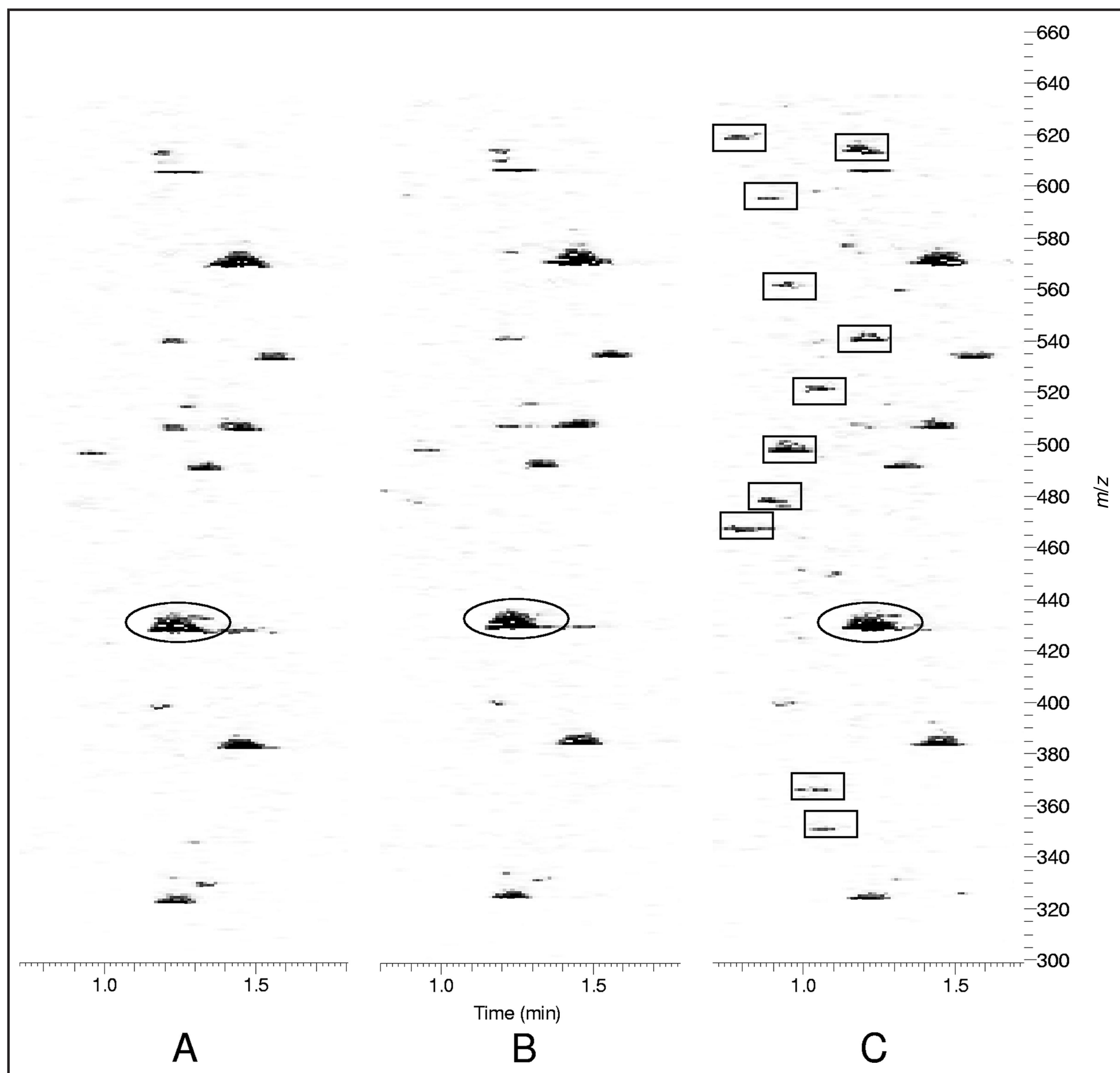

Fig. 4. Contour ion maps of precursor ion scan data without background subtraction.

The figure shows precursors of histidine immonium ion (*m/z* 113.1) and the Q1 scan from *m/z* 300–600. (A), Nondegrading human plasma at $t = 0$ min. (B), Nondegrading human plasma sample at $t = 30$ min. (C), Degrading human plasma sample at $t = 30$. The +3 charge state of the spiked, isotope-labeled peptide (*m/z* 435) is circled in (A), (B), and (C). Newly appearing peaks as a result of degradation are indicated with boxes in (C).

prior studies. For carefully prepared samples, correspondence of the 3-h and 18-h results has been strong evidence against substantial degradation of Ang I *(4, 17)*. Similarly, Fyhrquist et al. *(5)* reported that IEF analysis of radiolabeled Ang I that had been spiked into plasma indicated that it was stable for 1 h. In contrast, Derkx reported that Ang I was lost during 18-h incubations *(5)*. The work presented here, including the quantitative analysis of degradation and direct molecular characterization of Ang I degradation products from patient samples, conclusively demonstrates that Ang I is degraded in some patient samples, even under conditions that are currently believed to be optimal.

It should be pointed out that the observed mean losses we observed during extended generation should not suggest that 18-h PRA results are generally approximately 20%–25% lower than their corresponding 3-h result (Fig. 2). Simple application of the observed de-

Fig. 5. Precursor scan ion total ion current (background subtracted) and representative precursor scan spectrum for major peak.

Two coeluting degradation products with masses that match the experimental data are indicated with +2 and +3 charge states highlighted.

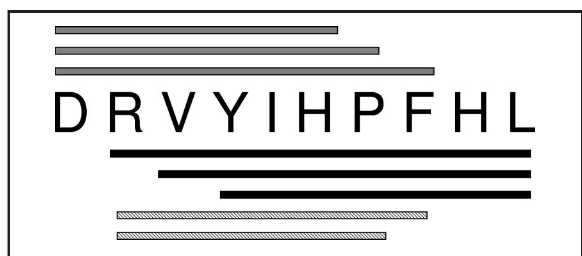

Fig. 6. Ang I degradation products identified in precursor ion scanning experiments and confirmed by targeted SRM.

▩, C-terminal fragments; ■, N-terminal fragments; ▧, internal fragments arising from N- and C-terminal processing.

gree of degradation is not justified without further knowledge of the kinetics of the degradation process. However, we believe our experimental approach to measuring degradation overestimates the difference between a 3-h and 18-h PRA result. In the degradation experiments presented here, all of the Ang I to be degraded was added at time zero. However, in a normally prepared sample, Ang I accumulates over time concomitant with degradation. Nevertheless, the general degradation of Ang I over time indicates that minimizing the generation time is an important goal for maintaining analytical integrity in the PRA assay. In this situation, the utility of LC-MS/MS sensitivity is apparent, since our assay offers acceptable performance for clinical samples with a 3-h generation.

Beyond the phenomena of mild degradation, our work has revealed a subpopulation of clinical samples that appear unsuitable for analysis with the Ang I accumulation strategy used in the PRA assay. Discovery of this subpopulation relied heavily on examining a large set of patient samples (>300) that focused on determining optimal generation times. It is important to note that the highly degrading population we have identified would normally be invisible to the RIA method as it is practiced, because both a 3- and 18-h result would be correspondingly low. Ongoing screening of sample remnants suggests that the frequency of these "high degradation" samples lies between 2% and 5% of the patient population. In this study, we have relied on deidentified clinical samples and have no information regarding the clinical status of any of the patients, although we presume they are diverse with respect to their baseline disease states. We have continued to investigate whether the observed high degradation phenotype is due to an in vivo or ex vivo phenomenon. In more recent work, second samples from patients demonstrating the high degradation phenotype were obtained several days after the initial measurement, and in each case, the degradation phenotype was repeated (n = 23). A patient sample that returns a very low PRA result while showing exceptionally high degradation activity prompts interesting questions regarding the clinical relevance and interpretation of the PRA result and associated ARR. It is possible that the ARR might not be a suitable screen for primary hyperaldosteronism in this context and that other approaches to rule out primary hyperaldosteronism should be considered when highly degrading samples are identified.

The exact nature of the proteases and peptidases that are responsible for the loss of Ang I in our samples remains unknown. Other studies have revealed candidates that have been characterized *(18–22)*. Physiologically, Ang I is a substrate for angiotensin-converting enzyme (ACE) 1, which functions to remove the two C-terminal amino acids (HL) to generate Ang II. A range of angiotensinase activities has been described, and recently ACE 2, a new angiotensin-converting enzyme, was described that generates Ang I 1–9 by the removal of a single C-terminal amino acid *(18)*. Ang III and Ang IV are N-terminal degradation products of Ang II that are generated by the activity of aminopeptidase A *(19–22)*. The physiological activities for many of these Ang I degradation products are not well understood, although the body of data highlights the fact that Ang I is a target for peptidase activity.

The major classes of proteases that act on Ang I and Ang II are known to be membrane bound and exert their activity at the endothelium. As metalloproteases, ACE 1, ACE 2, and aminopeptidase A are expected to be substantially inhibited by the use of EDTA as an anticoagulant, but we observed no inhibitory effect when testing supplementation of EDTA above concentrations used during sample collection. The use of non–angiotensin-related peptides to examine the specificity suggest that the activity is not entirely nonspecific. However, we note that the addition of protease inhibitors did not retard the degradation of these peptides either. This result is expected because many plasma proteases are known to act on multiple substrates.

In recent work, Yi et al. *(8, 9)* described the general stability of peptides in human serum and plasma. Our study, along with that by Yi et al., reemphasizes the undisputed effect that proteolytic activity in plasma and serum can have on clinical assays or proteomic-based biomarker discovery approaches. Yi et al. suggest that protease inhibited plasma would be a preferred sample type for proteome study, and they reference their observation of degradation. We found that the stability of Ang I and selected peptides in normal human plasma was generally quite good. However, in the sample population with strong propensity toward degradation, the activity was often dramatic and nonspecific. We believe that the persistence and magnitude of degradative processes in plasma is troublesome and deserves careful consideration when plasma is used to study protein/peptide analytes or biomarkers.

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