Human prorenin determination by hybrid immunocapture liquid chromatography/mass spectrometry: A mixed-solvent-triggered digestion utilizing D-optimal design

Ilja Burdman | Bjoern B. Burckhardt

Rationale: Human prorenin, representing the precursor of mature renin, has been discussed as a potential biomarker, e.g. in diagnosing primary hyperaldosteronism or diabetes-induced nephropathy. Currently, only immunoassays are available for prorenin quantification. As the similarity of prorenin to active renin impedes its accurate determination by immunoassay, mass spectrometry appears as an accurate alternative for differentiation of that protein.

Methods: Immunoaffinity purification plus a mixed-solvent-triggered digestion was combined with liquid chromatography/mass spectrometry (LC/MS) to enable a fast, sensitive, and less laboratory-intensive approach to the quantification of prorenin. Statistical experimental planning, which is known as Design of Experiments (DOE), was used to identify the optimal conditions for the generation of the signature peptides within a manageable number of experiments. The efficiency of the mixed-solvent-triggered digestion by trypsin was investigated using four different organic solvents: acetonitrile, acetone, tetrahydrofuran and methanol.

Results: By utilizing a D-optimal design, we found that the optimal mixed-solvent type for the generation of both signature peptides was acetonitrile at a concentration of 84% and an incubation temperature of 16°C. Using the mixed-solvent-triggered digestion, the procedure time allowed a fast analysis of active renin and prorenin with a short digestion time of 98 min. This optimized mixed-solvent-triggered digestion procedure was applied to detect renin and prorenin successfully in human plasma by the newly developed hybrid approach.

Conclusions: The identification of unique surrogates for human prorenin enabled the mass spectrometric differentiation between the two similar proteins. The novel hybrid approach successfully proved its ability to purify, detect and distinguish between prorenin and active renin in human plasma.
1 | INTRODUCTION

Protein analysis by liquid chromatography coupled to mass spectrometry (LC/MS) is becoming an important tool for discovery and quantification. The current focus is not only set on developing analytical methods for therapeutic proteins, but also as an emerging technique in biomarker research. Human prorenin, which seems to be an inactive precursor of mature renin, has gained attention since it was discovered that its concentration is correlated with the occurrence of different diseases. Some studies even suggest it as a potential biomarker, e.g. in diagnosing primary hyperaldosteronism or diabetes-induced nephropathy. In addition to the pathological influence of prorenin, it appears to have other important functions, as evidenced by its secretion in reproductive tissues like ovaries. Compared with active renin, which is usually analyzed for cardiovascular disease, prorenin is released continuously and is not influenced by acute stimuli such as a change in body position. Concerning the structural difference between prorenin and renin, a 43-amino-acid-long pro-segment that covers the catalytical center is the sole difference between their amino acid sequences. This pro-segment can change its conformation and uncover the proteolytical center, making the differentiation between active renin and prorenin difficult for many immunoassays. In most ligand-binding assays, reliable determination is only achieved either by proteolytical cleavage of the pro-segment after cryoactivation at 4°C overnight, or by a renin inhibitor that promotes the unfolding of the pro-segment. These assays are time-consuming and require calculative levels that may lead to inconsistent and inaccurate results.

The current challenges in the bioanalytical determination of low-abundance proteins using MS are commonly addressed by combining immunocapture with a customized digestion procedure. In the past, the commonly performed digestion procedures were often unsuitable due to their extensive preparation requirements and long durations. Several approaches, with their own benefits and limitations, have been developed to accelerate these procedures. The denaturing agents that are used in pure aqueous digestion procedures lead to difficulties with MS detection as well as possible interference with reversed-phase chromatographic separation, resulting in unpredictable peptide retention and elution properties. Moreover, unintentional reactivity of the used denaturing agents with the peptides of interest has been reported. Other agents such as guanidinium hydrochloride require an extended cleanup and subsequent dilution before tryptic digestion, making them unsuitable for low-abundance proteins.

Different types of organic solvents have shown their suitability for the denaturation of the protease-resistant proteins. These commonly applied organic solvents (e.g. acetonitrile and methanol) are characterized by a strong denaturation power. Moreover, these solvents have shown their compatibility with trypsin, particularly when modified (i.e. alkylated lysine residues). Several proteomic approaches have confirmed the usefulness of mixed aqueous–organic digestion for the acceleration and better production of surrogates for proteolytic-resistant proteins. However, it requires individual evaluation for each analyte of interest and depends on the structural particularities and amino acid sequence. For example, Shuford et al found organic solvents to be detrimental to the quantitative production of their peptides of interest.

To optimize digestion efficiency, the various factors that impact the generation of surrogates can be investigated either by analyzing one factor at a time or by using a statistical approach. By investigating each factor separately, the optimal set point might be missed. In addition, the determination of two or more surrogates makes this approach inappropriate. Therefore, a statistically planned experimental design following the quality-by-design approach allows for the analysis of different factors interacting with each other within a manageable number of experiments, and is described as the Design of Experiments (DOE). This study aims to investigate the suitability of a rapid mixed-solvent-digestion approach to prorenin determination using the DOE concept. The hybrid immunocapture LC/MS, which uses a label-free protein-level enrichment, should facilitate the reliable determination of prorenin and differentiate it from structurally related compounds. In addition, its applicability in human plasma must be confirmed.

2 | EXPERIMENTAL

A flowchart of the designed and performed experiments focusing on immunocapture, stability, organic digestion and applicability of the developed method is illustrated in Figure 1.

2.1 | Materials and chemicals

Human recombinant prorenin (≥85%) was supplied by Cayman Chemicals (Ann Arbor, MI, USA). TCPK-treated modified trypsin and Dynabeads® Protein G magnetic beads were purchased from Thermo Fisher Scientific (Rockford, IL, USA). Anti-prorenin antibodies were purchased from three different vendors (Genetex, Irvine, CA, USA; R&D Systems, Minneapolis, MN, USA; Molecular Innovations, Novi, MI, USA). Ammonium bicarbonate (>99.5%) was obtained from Sigma-Aldrich (Taufkirchen, Germany). Methanol optima® (LC-MS grade), tetrahydrofuran (HPLC grade), acetone (HPLC grade) and dimethyl sulfoxide (DMSO; p.a.) were supplied by Fisher Chemicals (Geel, Belgium). Water (LC-MS grade) was provided by Riedel-de Haen (Seelze, Germany), while formic acid (FA; 98%, p.a.) and acetonitrile (UHPLC grade) were supplied by Applichem (Darmstadt, Germany). Human blood plasma was collected from a healthy male volunteer in S-Monovette® K3 EDTA tubes (Sarstedt, Nuembrecht, Germany).
FIGURE 1  Flowchart of the designed and performed experiments carrying out important steps of method development focusing on immunocapture, stability, organic digestion, and applicability of the developed method [Color figure can be viewed at wileyonlinelibrary.com]
2.2 | Selection of signature peptides for determination

The mature part (chain; entry number P00797) plus the pro-peptide (43 amino acids) forms the so-called prorenin, which was extracted from the UniProt® Database (www.uniprot.org). The selection of the signature peptides was carried out by evaluation of the amino acid structure using protter® (http://ulo/github/io/Protter/), and possible tryptic cleavage sites were found. Peptides that contained cysteines were excluded to accelerate the sample pretreatment by avoiding reduction and alkylation steps. As an additional tool, Prospector® (prospector.ucsf.edu/prospector) was used to prove the desired in-silico digested peptides and simulate their fragmentation scheme to identify possible transitions. The selected peptides were considered as suitable for tryptic digestion by in-silico proteolysis and appraised by their amino acid structures.

As shown by Shuford et al, the analysis of peptide characteristics is essential for the development of a reliable assay by using the surrogate peptide approach.35 By performing a combined in-silico and experimental analysis of the surrogate peptide's properties (Peptide Analyzing Tool; Thermo Fisher Scientific), the peptide characteristics of the digested prorenin were assessed. The quantitative profiling of prorenin was conducted utilizing a sequential window acquisition of all theoretical fragment ion spectra (SWATH).

2.3 | Antibody screening for the hybrid approach

Human plasma is a highly complex matrix that makes the detection and determination of low-abundance proteins difficult. Therefore, immunocapture was chosen as a reliable and selective method for human prorenin purification. This study used antibodies against full-length prorenin. By using unselective as well as selective antibodies from different vendors, their capability to capture prorenin was examined. In total, seven different antibodies were screened for their suitability in a hybrid immunocapture LC/MS approach. Each antibody experiment was performed using an immobilization process on Dynabeads® protein G magnetic beads.

For the performance evaluation, 20 ng human recombinant prorenin was spiked into the plasma matrix (300 μL). The protein G beads combined with the antibodies were incubated for 1 h while vortexing in spiked plasma. A three-step washing process was performed: the samples were washed twice with a phosphate-buffered saline buffer (PBS 0.01% Tween 20) at the beginning, followed by a wash in a 25 mM ammonium bicarbonate (ABC) buffer to eliminate any interference by the Tween 20. All immunocapture complexes were digested completely in mixed solvents by adding 1 μg of modified trypsin and measured in triplicate using high-resolution TripleTOF mass spectrometry to confirm the identity of prorenin.

2.4 | Analyzing the stability of signature peptide residues and the pro-segment

The pro-segment structure of human prorenin can change its conformation, which is influenced by temperature and pH. To analyze the effect of this unfolding and its influence on the digestion procedure, high (40 °C) and low (10 °C) incubation temperatures were used to investigate the effect on tryptic cleavage in the mixed-solvent buffer following immunocapture.

The mature-part signature peptide and the pro-part signature peptide consist of a tryptophan residue that may be prone to different oxidized products. Kynurenine (+4 Da), hydroxytryptophan (+16 Da), and N-formylkynurenine/dihydroxytryptophan (+32 Da) represent different oxidative reaction products of the tryptophan residue. In addition, the methionine residue of the pro-part signature peptide was also considered for oxidation. Therefore, an investigation was performed into how potential oxidative agents such as acid and DMSO, which were used in the injection solvent mixture, influence these reactions.36,37 Possible oxidative products of both the mature-part and the pro-part signature peptides were calculated using the Prospector® tool (Table 1).

2.5 | Design of experiments

The design and evaluation of the experiments were accomplished using MODDE Pro© software (MKS Instruments AB, Malmo, Sweden, version 12.0). The D-optimal model was chosen as an experimental design due to the reduced costs and necessary number of experiments. In total, 114 experiments were required. To allow for a sufficient comparison with a full factorial design, we aimed for a G-efficiency of ≥0.6. In addition, the model fit (R²) and the Q² value, which estimate the predictive value of the design, were required to be ≥0.5. An appropriate design for overall model strength was defined as the difference between Q² and R² by values of below 0.3. Both signature peptides of human prorenin were incorporated into the model as a response, wherein the pro-part signature peptide was favored due to its uniqueness.

2.5.1 | D-optimal design for mixed-solvent digestion

Human recombinant prorenin (20 ng) was added to the 20 mM ABC buffer followed by the addition of 1 μg modified trypsin and adjusted to 100 μL with an organic solvent. The aqueous–organic trypsin ratio varied between the experiments depending on the corresponding DOE. Different organic solvents (tetrahydrofuran (THF), acetone, methanol, and acetonitrile) were used to analyze the influence of the organic solvent type and its concentration (60%–90%) on the digestion process. The incubation time ranged from 5 min to 2 h. The temperature was varied from 10 °C to 40 °C to investigate its impact on signature peptide generation. The lower temperature limit was chosen because of instrumental limitation and the upper limit was set...
at maximum of 40°C to limit imprecision by irregular solvent evaporation. Following the tryptic proteolysis, the mixed solvents were evaporated under a nitrogen stream and reconstituted in a mixture of 5% DMSO, 20% methanol, and 0.1% FA in water (v/v/v/v).

2.5.2 | Sweet-spot analysis of optimal digestion conditions

Due to the differences in the size as well as the amino acid composition and number of both investigated peptides, it was considered likely that distinct generation conditions for each signature peptide would be identified. Consequently, a sweet-spot analysis was performed in order to determine optimal settings with given predeterminants for a reliable generation of both surrogates. These calculations were made with 50,000 iterations at a resolution of 64 including all factors within the Monte Carlo simulation. For a robust and reliable result, the probability of failure, which estimates a false peak area prediction, was set to an acceptable limit of 0.5%.

2.6 | Applicability

Two aspects of the method's applicability in plasma were verified: First, the identified sweet-spot conditions for the digestion were confirmed following the immunocapture in neat solution as well as in plasma donated by a healthy male volunteer. To this purpose, 20 ng of human prorenin were spiked into either PBST buffer (neat solution) or 300 μL human plasma. The applied sweet-spot conditions concerning digestion using 84% acetonitrile, 16°C digestion temperature, and 98-min incubation time were measured in triplicate by LC/HRMS.

Second, the selectivity of the developed method was determined in blood samples containing both prorenin and active renin. For this experiment, 20 ng of human prorenin plus 20 ng of human active renin were added to the plasma samples (300 μL). These spiked levels were chosen following the pathophysiological prorenin/renin levels reported by Stoicescu et al.38 The experiment followed a two-step approach in which prorenin was captured first, followed by the immunopurification of active renin.39 The samples were digested using the sweet-spot conditions in triplicate and detected also by LC/HRMS.

In addition, the limit of detection (LOD) in plasma was estimated according to the criteria of the International Conference on Technical Requirements for Registrations of Pharmaceuticals for Human Use (ICH).40 The LOD was calculated by using the standard error and slope of the regression line of the specific calibration curve using the following expression:

\[ \text{LOD} = 3.3 \times \sigma/s \]

where \( \sigma \) is the standard error of the \( y \) intercept, used as standard deviation, and \( s \) is the slope of the regression line.

2.7 | Instrumentation and conditions

Two LC/MS systems were used for the analyses. The characterization experiments were conducted on a Shimadzu Nexera UHPLC system (Shimadzu Europe, Duisburg, Germany) coupled to a Sciex TripleTOF 6600 high-resolution mass spectrometer with an IonDrive TurboV®
electrospray ionization source (both from Sciex, Concorde, Canada) operated in positive ion mode. The settings applied for the TripleTOF mass spectrometer were as follows: curtain gas (N₂) pressure 35 psi, nebulizer gas (zero air) pressure 60 psi, heater gas (zero air) pressure 80 psi, ion spray voltage 5.5 kV, and interface heater temperature 300°C. The declustering potential was set to 54 V for the mature-part signature peptide and 30 V for the pro-part signature peptide, while the collision energy was adjusted to 54 eV for the mature-part signature peptide and 30 eV for the pro-part signature peptide. The investigated transitions are listed in Table 1. The tryptic peptide abundance was analyzed by a SWATH acquisition with 33 variable windows.

The DOE measurements were performed on a 1200 SL LC system (Agilent, Santa Clara, CA, USA) coupled to a 4000 triple quadrupole mass spectrometer (AB Sciex, Concorde, Canada) operated in positive ion mode. The following multiple reaction monitoring (MRM) transitions were employed: m/z 855.1 to 418 with 70 V declustering voltage and 36 eV collision energy for the mature-part signature peptide; and m/z 587.1 to 501.7 with 30 V declustering voltage and 25 eV collision energy for the pro-part signature peptide. The source parameters were set to curtain gas (N₂) pressure 30 psi, nebulizer gas (zero air) pressure 30 psi, heater gas (zero air) pressure 65 psi, ion spray voltage 5.5 kV, and interface heater temperature 450°C.

An XSelect CSH C18 column (130 Å, 3.5 μm, 3 mm × 150 mm; Waters, Milford, MA, USA) was used on both chromatographic systems to perform analyte enrichment and separation using a mixture of 1% DMSO, 0.1% FA in water (v/v/v) as mobile phase A and 1% DMSO, 0.1% FA in methanol (v/v/v) as mobile phase B.

2.8 | Data analysis

Data processing was performed using PeakView 2.2 and MultiQuant 3.0.2 (Sciex). BiopharmaView 3.0.2 software (Sciex) was utilized to carry out the in-silico tryptic digestion of human prorenin by downloading the FASTA file from UniProt (entry number P00797). The BiopharmaView 3.0.2 software was also used for evaluation of the digest. The assay was constructed by setting the digest agent as trypsin, and zero missed cleavages were allowed. By adjusting the processing setting to a mass-to-charge tolerance of 5 ppm, the extracted ion chromatogram (XIC) was set to 0.025 ppm and the MS/MS matching tolerance was adjusted to 0.03 Da.

3 | RESULTS AND DISCUSSION

3.1 | Optimal signature peptides for prorenin determination and their properties

The better differentiation of mature renin and prorenin (chain + pro-peptide) was achieved by identifying possible surrogates from the pro-segment ('pro-part signature peptide') and the chain structure ('mature-part signature peptide'). The in-silico digestion by the Prospector® tool revealed thirteen signature peptides for prorenin, of which two were unique for prorenin (confirmed by BLAST® analysis), and eleven for both active renin and the mature part of prorenin (Table S1, supporting information). Utilizing protter®, the beneficial positions of three signature peptides were identified (supporting information, S1).

The quantitative profiling of prorenin was conducted utilizing a sequential window acquisition of all theoretical fragment ion spectra (SWATH). The plotting of hydrophobicity and the determined amino acid sequence allowed for the identification of suitable candidates for the mixed-solvent approach. Both surrogates were chosen because of their beneficial amino acid structure for collision-induced fragmentation in the mass spectrometric approach. The peptides that were more hydrophobic were either too large (33 amino acids) or

FIGURE 2 Obtained peptide characteristics of digested prorenin. A, Quantitative profiling of different prorenin surrogates generated by the mixed-solvent approach using SWATH acquisition. For each of the three investigated organic solvents (acetonitrile, THF, and acetone), the hydrophobicity, the corresponding amino acid sequence ('sequence length'), and its abundance (size of dots) are plotted. The color allows for identification of product ions with different m/z values. The finally selected pro-part signature peptide is orange colored and the mature-part signature peptide is light blue colored. B, Excerpt of a chromatogram representing the obtained peptides after organic solvent digestion using acetonitrile. The final optimization of mass spectrometric signals identified VVFDTGSSNVVPSSK (mature-part signature peptide; light blue) and LGPEWSQPMK (pro-part signature peptide; orange) as the most intense ones [Color figure can be viewed at wileyonlinelibrary.com]
cleaved only to a small extent. The SWATH spectra revealed the abundance of the generated peptides (Figure 2). Although SWATH first found other peptides to be more abundant than the two selected surrogates, final mass spectrometric optimization revealed that the pro-part signature peptide and the mature-part signature peptide were the most intense.

The most intense surrogates that were named, ‘pro-part signature peptide’ (LGPEWSQPMK, unique for prorenin) and ‘mature-part signature peptide’ (VVFDTGSSNVWVPSSK, unique for prorenin and active renin within the plasma proteome), were analyzed by the DOE.

As opposed to the SISCAPA approach, in which a labeled version of the proteotypic peptide is added as isotope-labeled internal standard,16 in the here-presented setting the complete protein antigen is captured. This would have required a stable, labeled recombinant prorenin instead of only a unique peptide sequence as a suitable internal standard. However, for prorenin as well as for renin commercially available isotope-labeled full-length protein internal standards are lacking. The customized bioproduction would take several months, be expensive, and success would be uncertain. Therefore, a label-free approach was developed, which has been already shown to be promising.41

3.2 | Immunocapture

All seven screened antibodies of the three suppliers could capture human prorenin in a PBS buffer. However, only one antibody was effective enough to capture and differentiate between active renin and prorenin in human plasma. The elected antibody for the immunocapture in the hybrid approach also had inhibitory abilities that allowed it to avoid the conformational changes of the pro-segment of prorenin. This antibody was reported to bind selectively to the pro-segment (residues 32–39).15 By applying immunocapture with the inhibitory antibody against full-length prorenin, the purification was effective enough to avoid any interaction with plasma components that could cause ion suppression of the low-abundance proteins. Moreover, this immunocapture complex was suitable for organic solvent digestion and LC/MS measurement.

3.3 | Stability of signature peptides and pro-segment properties

The digestion temperature analysis revealed that the pro-part was more effectively cleaved at low temperatures (10°C), which could be explained by its ability to undergo conformational change when it was exposed to a cold environment.42 Although this conformational change was usually reported at 0°C and 4°C, the addition of organic solvents may change the behavior of the pro-segment and promote this structural conversion at higher temperatures (10°C). This property of the conformational change commonly caused the existing immunosorbent and radiometric assays to be imprecise. Here, however, this characteristic was used to enhance the response of the pro-part signature peptide.43

Neither oxidized nor di-oxidized residues were observed in the pro-part signature peptide sequence. This may be a result of the amino acid sequence and the cool digestion temperature, which protected this peptide from oxidative reactions. For the mature-part signature peptide, there was only one modification of the tryptophan amino acid observed, which was caused by high acidic content. A small amount of di-oxidized species (50 counts per second (cps)) of tryptophan (dihydroxy species) was observed, leading a mass increase of 32 Da (y6-fragment; Figure 3).

**FIGURE 3** Product ion spectrum of the mature-part signature peptide in its native and di-oxidized form. The di-oxidized species was detected as the y6-fragment of the mature-part signature peptide (overlaid red mass spectrum) [Color figure can be viewed at wileyonlinelibrary.com]
This investigation proved the stability of the surrogate peptide under the given conditions and therefore showed that it would facilitate human prorenin determination by hybrid LC/MS.

3.4 | Organic digestion D-optimal evaluation

The calculated D-optimal design was analyzed by a multiple linear regression model. This model was chosen to ensure the robustness of the generated data and had a G-efficiency of 0.79, which met the predetermined criteria. The desired statistical values of the analysis of variance (ANOVA) results indicated a $Q^2$ value of 0.86 and an $R^2$ value of 0.90 which fulfilled the predefined requirements for a good model fit and a reliable prediction of the applied model. These models also showed a good reproducibility value of 0.95.

By using the D-optimal model, 114 experiments (4 outlier experiments were excluded) were evaluated, comprising 35 designed runs with 3 center-points, all of which were measured in triplicate (Tables S2 and S3, supporting information).

3.4.1 | Effect of organic solvent type

The four organic solvents –THF, acetone, acetonitrile, and methanol – showed different results for the generation of both signature peptides. With the exception of acetone (and methanol for the pro-part signature peptide), all the organic solvents had a significant beneficial influence on the production of the analyzed surrogates (Figures 2 and 4). Each organic solvent allowed a proteolytic cleavage by modified trypsin that produced measurable levels of the mature-part signature peptide and the pro-part signature peptide.

The best results for the generation of both peptides were obtained using acetonitrile or THF. Acetone had a significant negative impact on the response of both surrogates, while methanol reduced the generation of the pro-part signature peptide (Table S2, supporting information).

3.4.2 | Effect of organic solvent concentration

The aqueous–organic solvent ratio played a crucial role in whether the pro-part or the mature-part was more efficiently generated. The optimal composition for efficient digestion varied depending on the type of organic solvent used. The individual effect of increasing the organic solvent concentration had a significant positive impact on the cleavage of the pro-part signature peptide (Figure 4(2)).

Concerning the individual effects regarding the mature-part signature peptide, the concentration did not have a significant influence on its generation. Nonetheless, a lower organic solvent ratio was slightly beneficial ($\alpha = 0.051$). However, the interactive effect of varying the concentration of the organic solvent type was the strongest effect on its generation. The interaction of a low organic solvent concentration (60%) and acetonitrile resulted in the most potent production of the mature-part signature peptide (mean 1.00e5 cps). Two of the analyzed organic solvents (THF and acetone) interacted positively with high organic contents; however, only THF had similar results to acetonitrile.

The pro-part signature peptide behaved differently by being generated at the highest concentration of almost all organic solvents; at 90% concentration, the intensities had a mean value of 2.30e4 cps using acetone, 2.47e4 cps using acetonitrile, and 2.27e4 cps using THF. Overall, the optimal solvent concentration for the pro-part signature peptide was the highest concentration used (90%). However, if the concentration interacted with the different organic solvent types, only methanol led to a significant decrease in pro-part signature peptide generation ($\alpha < 0.01$). For the mature-
part, a high organic solvent concentration and a high temperature were unsuitable for cleavage (Figure 4(1) and Table S2, supporting information).

Therefore, the optimal settings for both peptides were determined using acetonitrile as the organic solvent, which showed a reliable generation at the lowest (60%) and highest (90%) organic solvent content employed. The THF concentration displayed the opposite characteristic, with the best performance found at a high organic content (90%). In the end, acetonitrile outperformed the other organic solvents when interacting with other factors (Table S2, supporting information).

3.4.3 | Effect of digestion temperature

The optimal digestion temperature, ranging from 10°C to 40°C, was strongly correlated with the type of organic solvent. The optimal temperature for the mature-part signature peptide was 40°C, whereas generation of the pro-peptide part was favored by cooler temperatures (10°C). This can be explained by the better unfolding of the pro-segment, which displayed an ability to change its conformation under the influence of cold temperature.

As an individual effect, elevated temperature was not favorable in generating the signature peptides. In the case of the interactive effects, increased temperature was only significantly advantageous for THF and acetonitrile as the organic solvent for the mature-part signature peptide (Figure 4(1)). The mature-part signature peptide showed the best response at 10°C incubation temperature (mean 9.04e4 cps) in methanol, followed by THF (mean 8.12e4 cps), while the lowest responses were observed in acetone and acetonitrile, with mean values of 7.20e4 and 7.12e4 cps, respectively. An additional increase of 10°C was still beneficial for methanol (1.08e5 cps), while the digestion intensity in THF and acetonitrile increased by 31% and 40%, respectively. An increase in incubation temperature up to 30°C was shown to be advantageous for acetonitrile and THF, with respective mean values of 1.24e5 and 1.28e5 cps. A smaller effect was seen for methanol and acetone, with respective mean values of 1.22e5 and 1.04e5 cps. A temperature of 40°C resulted in the highest intensity of the mature-part signature peptide for all organic solvents, the highest being THF and acetonitrile each with a mean intensity of 1.45e5 cps.

The lower temperatures better promoted generation of the pro-part signature peptides (Figure 4(2)). With regard to the acetonitrile-water solvent system for example, generation of the pro-part signature peptide was improved at the lower temperatures of 20°C and 30°C (with mean values of 1.66e4 and 1.65e4 cps, respectively) compared with at 40°C (mean 1.56e4 cps; Table S2, supporting information).

3.4.4 | Sweet-spot analysis for the mature-part and pro-part signature peptides

Prorenin uniqueness was defined by its pro-peptide, which is completely absent in the active renin. This property is beneficial for analyzing the selectivity of antibodies and ensuring the differentiation between the two proteins. The optimal conditions for the generation of both surrogate peptides were a concentration of 84% acetonitrile, a digestion temperature of 16°C, and an incubation time of 98 min. The Monte-Carlo-calculated optimal set point intensity was predicted as 1.2e5 cps for the mature-part signature peptide and 3.7e4 cps for the pro-part signature peptide. The robustness of the defined sweet-spot was calculated at 0.17% probability of failure.

4 | APPLICABILITY

First, the evaluated sweet-spot conditions were confirmed following the immunocapture in neat solution as well as in human plasma. The conditions resulted in detectable levels of the pro-part and mature-part signature peptides in human plasma. Second, by applying the optimal conditions within the hybrid approach, a reliable determination of the spiked recombinant human prorenin was achieved. Prorenin as well as active renin were successfully captured by the selective antibodies and detected by LC/MS. The identities of both surrogates were confirmed by the typical fragmentation scheme of γ- and b-fragments utilizing high-resolution MS. For the mature-part signature peptide, the y4-fragment was the most intense, caused by the proline fragmentation side of the PSSK fragment. In the case of the pro-part signature peptide, the most intense fragments were y8, representing the fragmentation on the proline side of the PEWSQPMK part, and y3, with the other proline residue of the PMK part (Figure 5). The collision energy was optimized so that the mature-part signature peptide was the most intense for the active renin and the pro-part signature peptide had the highest intensity for the pro-segment of prorenin (supporting information, S2). The absence of the pro-part signature peptide in the active renin digests underlines the selectivity of the hybrid immunocapture LC/MS method presented herein. The final workflow showing all steps and duration of the complete assay is illustrated in the supporting information (S3).

This assay optimization provided a LOD of 110 pg/mL for prorenin as well as a LOD of 26 pg/mL for active renin. These limits appear acceptable in the context of reported protein levels in human plasma (plasma prorenin mean ± SD of 2130 ± 250 pg/mL for 38-week-old healthy neonates44; plasma active renin mean ± SD of 226 ± 58 pg/mL for 16 h to 1-month-old healthy neonates45; plasma prorenin median of 552 pg/mL [interquartile range; IQR: 297–1097 pg/mL] for cardiovascular disease adults46; plasma active renin median of 46.8 pg/mL [interquartile range; IQR: 31.8–91.8 pg/mL] for cardiovascular disease adults47).

Although a full validation has not yet been conducted, the precision was determined in triplicate for both surrogates. A repeatability (coefficient of variance (CV)) of 1% for the mature-part signature peptide and 7% for the pro-part signature peptide was obtained.

In addition to the detection of pure prorenin, the developed LC/MS method can also differentiate between the open/unfolded...
forms of prorenin and renin. This is a main advantage over available immunoassays. Even if the selective antibodies identified within the presented work were used to develop better immunoassays, these assays still lack the ability to differentiate between the open form of prorenin and active renin. This would be especially pronounced if the samples from patients with highly elevated plasma levels of renin and prorenin were analyzed. For example, pharmacotherapy with so-called direct renin inhibitors such as aliskiren in the treatment of hypertension promotes the unfolding of the pro-segment, making this prorenin invisible for the prorenin ELISA and therefore it may be falsely detected as renin by the active renin immunoassay. The developed assay can still differentiate this state of prorenin from active renin through the pro-part signature peptide which is absent in active renin. Overall, this development is a good illustration of the symbiotic effects obtained by applying the hybrid LC/MS approach. The immunocapture provides the required sensitivity that current MS approaches lack to determine endogenous levels in complex matrices, while the mass spectrometer ensures the necessary selectivity.

To the best of our knowledge, this is the first time that a LC/MS method for the determination of endogenous levels of prorenin has been described. In addition to its benefits in overcoming the inability of the immunoassay to properly differentiate between prorenin and related compounds such as the active renin, the overall workflow is less complicated than in most currently available approaches. First, the time for the digestion part of the assay was reduced by 98 min and an overnight digestion is no longer necessary. Second, a laboratory equipped with an LC/MS system can easily implement this method into routine measurements without additional equipment. Since the sample purification of whole digest (e.g., by solid-phase extraction (SPE)) is not necessary, in comparison with common purification methods, the approach is more cost-effective and time-effective (the entire determination process lasts 3 h). Third, the usefulness of the assay for clinical application to samples collected from vulnerable population (e.g., pediatrics) is ensured. In particular, the advantage of being able to reuse blood plasma after immunocapture for the determination of other parameters is beneficial to overcome ethical constraints concerning the limited blood volumes in those populations.

5 | CONCLUSIONS

This assay enabled the determination of human prorenin on triple quadrupole and QTOF systems by a simplified and fast hybrid protocol using immobilized, highly-specific antibodies for immunocapture and acetonitrile at high concentration as an organic denaturant. Due to the simultaneous determination of a signature peptide cleaved from the pro-segment and a signature peptide generated from the mature renin, two-level specificity was ensured.

ACKNOWLEDGEMENTS

Open access funding enabled and organized by Projekt DEAL.

PEER REVIEW

The peer review history for this article is available at https://publons.com/publon/10.1002/rcm.8932.

ORCID

Bjoern B. Burckhardt https://orcid.org/0000-0002-1782-9937

REFERENCES

1. Peng X, Liu B, Li Y, et al. Development and validation of LC–MS/MS method for the quantitation of infliximab in human serum. Chromatographia. 2015;78(7–8):521–531. https://doi.org/10.1007/s10337-015-2866-2
2. Iwamoto N, Shimada T, Terakado H, Hamada A. Validated LC–MS/MS analysis of immune checkpoint inhibitor Nivolumab in human plasma using a Fab peptide-selective quantitation method: Nano-surface and molecular-orientation limited (nSMOL) proteolysis. J Chromatogr B Analyt Technol Biomed Life Sci. 2016;1023–1024:9-16. https://doi.org/10.1016/j.jchromb.2016.04.038
3. Pang JX, Ginanni N, Dongre AR, Hefta SA, Opiteck GJ. Biomarker discovery in urine by proteomics research articles. J Proteome Res. 2002;1(2):161-169. https://doi.org/10.1021/pr015518w

4. McDonald WH, Li JRY. Shotgun proteomics and biomarker discovery. Dis Markers. 2002;18(2-99.105.

5. Zhang H, Gu H, Shipkova P, et al. Immunooaffinity LC-MS/MS for quantitative determination of a free and total protein target as a target engagement biomarker. Bioanalysis. 2017;9(20):1573-1588. https://doi.org/10.4155/bio-2017-0152

6. Torsetnes SB, Levbak SG, Claus C, et al. Immunocapture and LC-MS/MS for selective quantification and differentiation of the isozymes of the biomarker neuron-specific enolase in serum. J Chromatogr B Anal Technol Biomed Life Sci. 2013;929:125-132. https://doi.org/10.1016/j.jchromb.2013.04.010

7. Naruse M, Wasada T, Naruse K, Yoshimoto T, Omon Y, Demura H. Pathophysiological significance of plasma total renin and prorenin in patients with diabetes mellitus. Endocr J. 1995;42(2):225-233. https://doi.org/10.1507/endocrj.42.225

8. Berge C, Courand PY, Harbaoui B, et al. Decreased plasma prorenin levels in primary aldosteronism: Potential diagnostic implications. J Hypertens. 2015;33(1):118-125. https://doi.org/10.1097/HHJ.0000000000000367

9. Sealey JE, Glorioso N, Itskovitz J, Laragh JH. Prorenin as a marker of target engagement biomarker. Bioanalysis. 2005;90(1):1041-1046. https://doi.org/10.1016/0002-9343(86)90402-X

10. Suzuki F, Hayakawa M, Nakagawa T, et al. Human prorenin has "gate and handle" regions for its non-proteolytic activation. J Biol Chem. 2003;278(25):22121-22222. https://doi.org/10.1074/jbc.M302579200

11. Hsueh WA, Baxter JD. Human prorenin. Hypertension. 1991;17(4):469-477. https://doi.org/10.1161/01.HYP.17.4.469

12. Menard J, Guyene T-T, Peyrard S, Azizi M. Conformational changes in prorenin during renin inhibition in vitro and in vivo. J Hypertens. 2006;24(3):529-534. https://doi.org/10.1097/01.jhj.0000209989.59230.2e

13. Tu W, Eckert GJ, Pratt JH, Jan Danser AH. Plasma levels of prorenin and renin in blacks and whites: Their relative abundance and associations with plasma aldosterone concentration. Am J Hypertens. 2012;25(9):1000-1004. https://doi.org/10.1038/ajh.2012.83

14. Derix FHM, De Bruin RIA, Van Gool JMG, et al. Clinical validation of renin monoclonal antibody-based sandwich assays of renin and prorenin, and use of renin inhibitor to enhance prorenin immunoreactivity. Clin Chem. 1996;42(7):1051-1063.

15. Krop M, Van Gool JMG, Day D, Hollenberg NK, Danser AHJ. Evaluation of a direct prorenin assay making use of a monoclonal antibody directed against residues 32-39 of the prosegment. J Hypertens. 2011;29(11):2138-2146. https://doi.org/10.1097/HHJ.0b013e3282d1b798

16. Anderson NL, Anderson NG, Haines LR, Hardie DB, Olafson RW, Pearson TW. Mass spectrometric quantitation of peptides and proteins using stable isotope standards and capture by anti-peptide antibodies (SISCAPA). J Proteome Res. 2004;3(2):235-244. https://doi.org/10.1021/pr0304806h

17. Figyels D. A critical review of trypsin digestion for LC-MS based proteomics. Proteomics. 2013;13(8):1231-1232. https://doi.org/10.1002/pmic.201370077

18. ProC JL, Kuzyk MA, Hardie DB, et al. A quantitative study of the effects of chaotrophic agents, surfactants, and solvents on the digestion efficiency of human plasma proteins by trypsin. J Proteome Res. 2010;9(10):5422-5437. https://doi.org/10.1021/pr100655u

19. Wang S, Zhang L, Yang P, Chen G. Infrared-assisted trypsinic proteolysis for peptide mapping. Proteomics. 2008;8(13):2579-2582. https://doi.org/10.1002/pmic.200800086
36. Perdivara I, Deterding LJ, Przybyski M, Tomer KB. Mass spectrometric identification of oxidative modifications of tryptophan residues in proteins: Chemical artifact or post-translational modification? *J Am Soc Mass Spectrom*. 2010;21(7):1114-1117. https://doi.org/10.1016/j.jasms.2010.02.016

37. Van De Weert M, Lagerwerf FM, Haverkamp J, Heerma W. Mass spectrometric analysis of oxidized tryptophan. *J Mass Spectrom*. 1998;33(9):884-891.

38. Stoicescu M, Csepento C, Muţiu G, Bungău S. The role of increased plasmatic renin level in the pathogenesis of arterial hypertension in young adults. *Rom J Morphol Embryol*. 2011;52(SUPPL. 1):419-423.

39. Burdman I, Burckhardt BB. A concept to make low-abundance endogenous renin accessible to mass spectrometry: A multistep experimental design approach. *J Chromatogr B*. 2019;1134–1135 (August):121856. https://doi.org/10.1016/j.jchromb.2019.121856

40. EMA. ICH Topic Q 2 (R1) Validation of Analytical Procedures: Text and Methodology. Prescrire International. https://www.ema.europa.eu/en/documents/scientific-guideline/ich-q-2-r1-validation-analytical-procedures-text-methodology-step-5_en.pdf. Published 2011.

41. Al Shweiki MHDR, Mönchgesang S, Majovsky P, Thieme D, Trutschel D, Hoehenwarter W. Assessment of label-free quantification in discovery proteomics and impact of technological factors and natural variability of protein abundance. *J Proteome Res*. 2017;16(4):1410-1424. https://doi.org/10.1021/acs.jproteome.6b00645

42. Pitarresi TM, Rubattu S, Heinrikson R, Sealey JE. Reversible cryoactivation of recombinant human prorenin. *J Biol Chem*. 1992;267(17):11753-11759.

43. Campbell DJ, Nussberger J, Stowasser M, et al. Activity assays and immunoassays for plasma renin and prorenin: Information provided and precautions necessary for accurate measurement. *Clin Chem*. 2009;55(5):867-877. https://doi.org/10.1373/clinchem.2008.118000

44. Terada T, Urushihara M, Saijo T, Nakagawa R, Kagami S. (pro)renin and (pro)renin receptor expression during kidney development in neonates. *Eur J Pediatr*. 2017;176(2):183-189. https://doi.org/10.1007/s00431-016-2820-9

45. Blazy I, Guillot F, Laborde K, Dechaux M. Comparison of plasma renin and prorenin in healthy infants and children as determined with an enzymatic method and a new direct immunoradiometric assay. *Scand J Clin Lab Invest*. 1989;49(5):413-418.

46. Yoshida G, Kawasaki M, Murata I, et al. Higher plasma prorenin concentration plays a role in the development of coronary artery disease. *Biomark Res*. 2015;3(1):1-7. https://doi.org/10.1186/s40364-015-0044-1

47. Tomaschitz A, Pilz S, Ritz E, et al. Associations of plasma renin with 10-year cardiovascular mortality, sudden cardiac death, and death due to heart failure. *Eur Heart J*. 2011;32(21):2642-2649. https://doi.org/10.1093/eurheartj/ehr150

**SUPPORTING INFORMATION**

Additional supporting information may be found online in the Supporting Information section at the end of this article.