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Development and validation of an LC-MS/MS method for the simultaneous quantitation of angiotensin (1–7), (1–8), (1–9) and (1–10) in human plasma

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ABSTRACT

Cardiovascular diseases have cast a significant negative impact on the lives of millions worldwide. Over the years, extensive efforts have been dedicated to enhancing diagnostic and prognostic tools for these diseases. A growing body of evidence indicates that the angiotensin convertase enzyme (ACE) and the angiotensin convertase enzyme 2 (ACE2), and angiotensin peptide levels could hold a pivotal role in assisting clinicians with the management of cardiovascular conditions, notably hypertension and heart failure. However, despite the considerable body of knowledge in this domain, a void remains in the field of analytical methodologies for these molecules. In this study, we present a fully validated LC-MS/MS method for the precise quantitation of plasma angiotensin (1–7), (1–8), (1–9), and (1–10), following the guidelines set by the Clinical and Laboratory Standards Institute (CLSI). Our method not only enables the accurate quantification of angiotensin peptides but also provides a means to assess ACE and ACE2 activity. Remarkably, our method achieved a Lower Limit of Measurement Interval (LLMI) as low as 5 pg/mL. This has enabled the detection of angiotensin (1–7), (1–8), (1–9) and (1–10) and the accurate quantitation of angiotensin (1–7), (1–8) and (1–10) in all analyzed groups, including healthy controls, patients with high blood pressure, and patients with chronic kidney disease. To our knowledge, our method represents the most sensitive approach allowing for simultaneous quantitation of these four angiotensin peptides. A distinct advantage of our method, when compared to immunoassays, is its high sensitivity combined with comprehensive chromatographic separation of all currently known angiotensin peptides. This combination translates to an exceptional level of selectivity, underscoring the value and potential of our methodology in advancing cardiovascular disease research.

1. Introduction

The Renin-Angiotensin-Aldosterone system (RAAS) orchestrates a complex cascade involving a network of enzymes and vasoactive peptides [1]. Its ultimate product, angiotensin II (Ang (1–8)), emerges from angiotensin I (Ang (1–10)) through the catalytic action of angiotensin-converting enzyme (ACE) [2,3]. This precursor, Ang (1–10), stems from hepatic angiotensinogen, undergoing proteolytic processing

Abbreviations: ACE, Angiotensin Convertase Enzyme ; ACE 2, Angiotensin Convertase Enzyme 2 ; ANG, Angiotensin ; ANOVA, single-nested analysis of variance ; AUC, Area under the curve ; BSA, Bovin Serum albumin ; CKD, Chronic kidney disease ; CTRL, Control ; CV, Coefficient of variation ; ESI, Electrospray ionization ; FA, Formic Acid ; HBP, High blood pressure ; LC-MS/MS, Liquid chromatography coupled to tandem mass spectrometry ; LLMI, Lower limit of measurement interval ; LOD, Limit of detection ; MAS Receptor, Mitochondrial assembly receptor ; MRM, Multiple reaction monitoring ; MS, Mass spectrometry ; MU, Measurement uncertainty ; PBS, Phosphate buffer saline ; QC, Quality control ; RAAS, Renin-Angiotensin-Aldosterone system ; SIL-IS, Isotopically labelled internal standard ; SPE, Solid-phase extraction.

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[1] Equal contribution: These authors contributed equally to this work. The selection of the first author in the list was determined using the "flip a coin" method. However, both authors retain the right to place themselves as the first author in their respective curriculum vitae.

[2] Equal contribution: These authors contributed equally to this work.

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catalysed by renal renin [4]. Cascade is represented in Fig. 1. Operating as a major pillar of cardiovascular and respiratory homeostasis [5], the RAAS plays a pivotal role. However, in pathological scenarios, elevated Ang (1–8) levels can instigate vasoconstriction, sodium retention, oxidative stress, fibrosis, cellular proliferation and inflammation [6–9]. Such deviations from equilibrium within this system may contribute to the emergence of cardiovascular diseases and kidney injuries (5–6,10).

In 2000, the analogous counterpart of ACE, known as ACE2, was discovered by Donoghue *et al.* Notably, ACE2 serves as a counterbalance to the deleterious effects of Ang (1–8) by enzymatically cleaving it into Ang (1–7) [11]. Beyond this pivotal role, ACE2 also facilitates the proteolytic cleavage of Ang (1–10) into Ang (1–9), which subsequently undergoes further enzymatic action to yield Ang (1–7), catalysed by ACE [3]. Nevertheless, it is important to note that this cleavage of Ang (1–10) remains approximately 400-fold less efficient than the conversion of Ang (1–8) into Ang (1–7) [3]. The concerted action of ACE2, enabling the conversion of Ang (1–8) into Ang (1–7), precipitates the activation of the mitochondrial assembly receptor (MAS receptor), thereby instigating vasodilation, apoptosis, natriuresis, and diuresis—manifesting as an array of cardioprotective effects [6]. This orchestrated response by Ang (1–7) ultimately leads to decrease blood pressure and norepinephrine levels, concurrently enhancing cardiac baroreflex sensitivity—contributing to protection against further heart failure.

Multiple investigations have substantiated the observation of reduced plasma levels of ACE2 and Ang (1–7) in patients afflicted with various cardiovascular damages. These conditions encompass pulmonary vascular permeability, pulmonary oedema, acute respiratory distress syndrome, atherosclerosis, hypertension, cardiac hypertrophy, and heart failure [12–14].

Notably, despite the considerable body of knowledge in this domain, a void remains in the field of analytical methodologies. Presently, no validated mass spectrometry method exists for the simultaneous quantitation of Ang (1–10), Ang (1–8), Ang (1–7), and Ang (1–9) at exceedingly low concentrations. The significance of reaching low concentrations cannot be overstated, especially given that Ang (1–7) and (1–9) exist in plasma at levels below 50 pg/mL. Some commercially available immunoassays were developed. Some of them necessitate a sample extraction such as a solid phase extraction or a chromatographic separation before the assays as multiple angiotensin peptides with very similar sequences coexist in the plasma. However, even with the extraction step, immunoassays remain poorly reproducible with each other and concerns about their specificity were made [15]. LC-MS/MS methods were developed for the quantitation of several angiotensin peptides. Few of them display very low LLMI/LOD however, no validation data were provided to support these LLMI/LOD [4,16–19]. LC-MS/MS methods were reported in Table 1.

Thus, the aim of our study was to address this gap by devising innovative methodologies to comprehensively analyse peptide profiles extracted from patients grappling with diverse pathologies. The ultimate aim of this endeavour is to prognosticate potential negative outcomes in patients suffering from cardiovascular diseases.

2. Materials and methods

2.1. Reagents and instruments

Angiotensin standards, including (1–10), (1–7), and (1–9), were procured from Merck KGaA (Darmstadt, Germany), while the standard for angiotensin (1–8) was obtained from Anaspec (California, USA). Isotopically labeled angiotensins (SIL-IS) were acquired from Eurogentec S.A. (Seraing, Belgium) for (1–7) (H-DRV(U-$^{13}C_5$ ^{15}N)YIHP-OH) and (1–9) (H-DRV(D_8)YIHPF(Ring-D_5)H-OH), and from Anaspec (California, USA) for (1–10) (H-DRV(D_8)YIHPF(Ring-D_5)HL-OH)and (1–8) (H-DRVYI($^{13}C_6$, ^{15}N)HPF-OH).

LC-MS grade methanol, acetonitrile, and water were sourced from Biosolve (Dieuze, France). Ammonium hydroxide (NH_4OH), Bovine

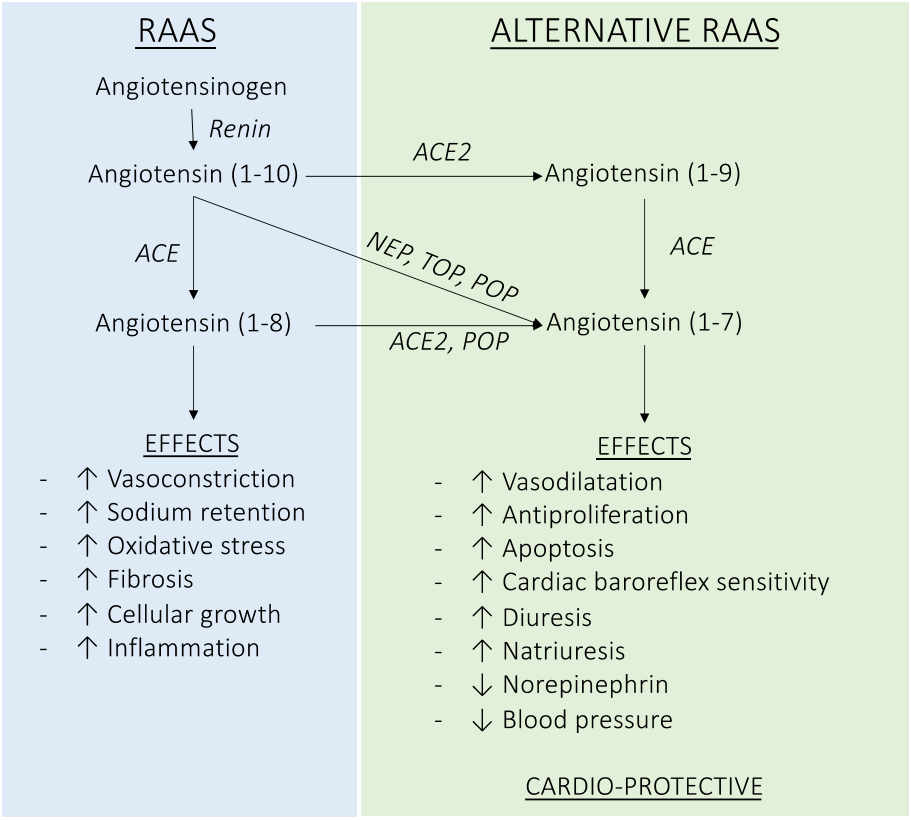

Fig. 1. RAAS and alternative RAAS cascade.

Table 1
Preexisting LC-MS/MS methods for the quantitation of Ang (1–7), (1–8), (1–9) and (1–10).

Authors	Peptides	Incubation	LLMI	Validation
Bernstone et al. 2021	Ang (1–8)	45 min 37 °C	10 pg/mL	V
Binder et al 2019	Ang (1–10) Ang (1–8) Ang (1–5) Ang (1–7)	30 min 37 °C	4 pg/mL (Ang 1–10) 2 pg/mL (Ang 1–7) 2 pg/mL (Ang 1–8)	/
Basu et al. 2017	Ang (1–10) Ang (1–8) Ang (2–8) Ang (3–8) Ang (1–9) Ang (1–7) Ang (1–5) Ang (2–7) Ang (3–7) Ang (2–10)	30 min 37 °C	3 pg/mL (Ang 1–9) 2.5 pg/mL (Ang 1–10 −2 pg/mL (Ang 1–8, 1–7)	/
Schulz et al. 2014	Ang (1–8)	/	6 pg/mL	V
Cui et al. 2007	Ang (1–8) Ang (2–8) Ang (3–8) Ang (1–7)	0–60 min 37 °C	25 pg/mL (Ang 1–7, 1–8)	V

Serum Albumin (BSA), and LC-MS grade formic acid (FA) were respectively obtained from Merck KGaA (Darmstadt, Germany), and Fischer Chemicals (Pittsburgh, PA, USA). Cow plasma, utilized as a surrogate matrix, was freshly collected by the Veterinary Faculty of the University of Liège. Phosphate Buffered Saline (PBS) was procured from Thermo Fisher (Kandel, Germany).

2.2. Standards, calibration, and quality controls

The standards and SIL-IS were dissolved in a solution containing H_2O, 5% ACN, 0.4% FA, and 0.1% BSA (w/w). Aliquots of 100 µL were transferred to 1.5 mL Eppendorf vials, centrifugated, and subsequently stored at −80 °C. Working and spiking solutions were prepared using H_2O, 5% ACN, 0.4% FA, and 0.1% BSA (w/w). All solutions were prepared in Protein LoBind Eppendorf tubes. Prior to making dilutions, the purity of all standards was carefully considered to ensure the accuracy of our measurements.

Regarding the calibration curves, a set of 8 calibrators was prepared to achieve the following concentration levels: 5 pg/mL, 10 pg/mL, 25 pg/mL, 50 pg/mL, 100 pg/mL, 250 pg/mL, 2.5 ng/mL, and 5 ng/mL. At these 8 points, we added a blank and a double blank.

As no stripped matrix was available, cow plasma was diluted by 50% with PBS and 0.1% BSA (w/w) to serve as a surrogate matrix. Structural differences exist between human and bovine angiotensins and our method is specifically designed to recognize and quantify the human forms of angiotensins. This was done due to the presence of interference at the retention time and in both transitions of Ang (1–10) and Ang (1–7) in undiluted cow plasma. For calibration, 0.9 mL of cow plasma diluted with PBS and 0.1% BSA (w/w) was spiked with 100 µL of calibration solutions of varying concentrations (5 pg/mL, 15 pg/mL, 30 pg/mL, 75 pg/mL, 150 pg/mL, 250 pg/mL, 500 pg/mL, 2 ng/mL, 4 ng/mL and 8 ng/mL). These calibrators were prepared in 2 mL Protein LoBind Eppendorf tubes. The calibration curve was built by correlating the peak area ratio of the native standard to the IS, employing a weighted ($1/x^2$) linear least squares regression.

Quality controls (QCs) were established by pooling human plasma samples into three distinct pools, followed by spiking the pools at three different concentrations (10 pg/mL, 100 pg/mL, and 4 ng/mL). Subsequently, the pooled samples were divided into 1 mL aliquots in Protein LoBind Eppendorf tubes and frozen at −80 °C.

2.3. Sample preparation

Plasma samples were subjected to centrifugation for 5 min at 3500 RPM at room temperature. Subsequently, 1 mL of the sample, quality control (QC), or calibrator was carefully transferred to 2 mL Protein LoBind Eppendorf tubes. To these samples, 50 µL of SIL-IS at a concentration of 25 ng/mL was added. After thorough homogenization, 500 µL of a mixed solution comprising H_2O, 1.25% NH_4OH (v/v) was introduced to the samples, calibrators, and QCs.

For the purification and concentration of samples, OASIS MAX 96-well plate 30 mg solid-phase extraction (SPE) plates from Waters were employed. SPE was conditioned with 1000 µL of methanol followed by 1000 µL of H_2O, 1.25% NH_4OH. Diluted samples were loaded and washed by 1000 µL of H_2O, 1.25% NH_4OH and H_2O, 30% ACN. Elution required 2 × 125 µL of H_2O, 50% ACN, 0.4% FA. After drying with nitrogen at a flow of 0,2mL/min until complete evaporation, samples were reconstituted with 100 µL of H_2O, 10% ACN with 0.4% FA and filtered. The detailed procedure for sample preparation is elaborated in Fig. 2.

2.4. LC-MS/MS analysis

Chromatographic separation was accomplished using a Nexera X2 UPLC system from Shimadzu (Shimadzu Corporation, Kyoto, Japan). A Luna Omega® C18 100 Å core–shell column measuring 100 × 2.1 mm, featuring a particle size of 1.6 µm and sourced from Phenomenex (Torrance, CA, USA), was employed. The column was thermostatted at 55 °C for consistent performance. The elution gradient was initiated at 5% mobile phase B, ramped to 25% B over a duration of 7 min, then escalated to 80% B, held for 5 min, and subsequently re-equilibrated to 5% B for 5 min. Mobile phases A and B were composed of water and acetonitrile, respectively, each containing 0.4% formic acid. The LC-MS/MS analysis was carried out using a flow rate of 0,4 mL/min, and an injection volume of 50 µL.

The detection was carried out using an AB Sciex (CA, USA) QT6500 mass spectrometer, incorporating Triple Quadrupole and Linear Trap analyzers. Samples were ionized through electrospray ionization in positive mode (ESI +). Unit resolution was employed in both Q1 and Q3 quadrupoles to facilitate the selection of two multiple reaction monitoring (MRM) transitions, which were utilized for both native and SIL-IS angiotensins. To achieve the requisite sensitivity within the low pg/mL range, all MS parameters were optimized. The specific MRM transitions, along with the corresponding compound and source parameters, are comprehensively detailed in Table 2.

2.5. Analytical validation

The design of the validation process was informed by the guidelines provided by the Clinical and Laboratory Standards Institute (CLSI-C62A). Quality control samples (QCs), spanning from the lowest to the highest concentration levels (V1-V8), were prepared and subjected to analysis in quadruplicate across a span of 5 consecutive days.

2.5.1. Selectivity

To ensure the chromatographic separation of the diverse angiotensin variants naturally present in the human body (angiotensin (1–5), (2–10), (2–8), (2–7), (3–8), (3–7), (1–7), (1–8), (1–9), and (1–10)), a comprehensive verification process was undertaken. Given that most of these variants exist in human plasma at very low concentrations, a concentration step was deemed necessary. To address this, a pool of residual human plasma samples (n = 42) was prepared, and each sample was

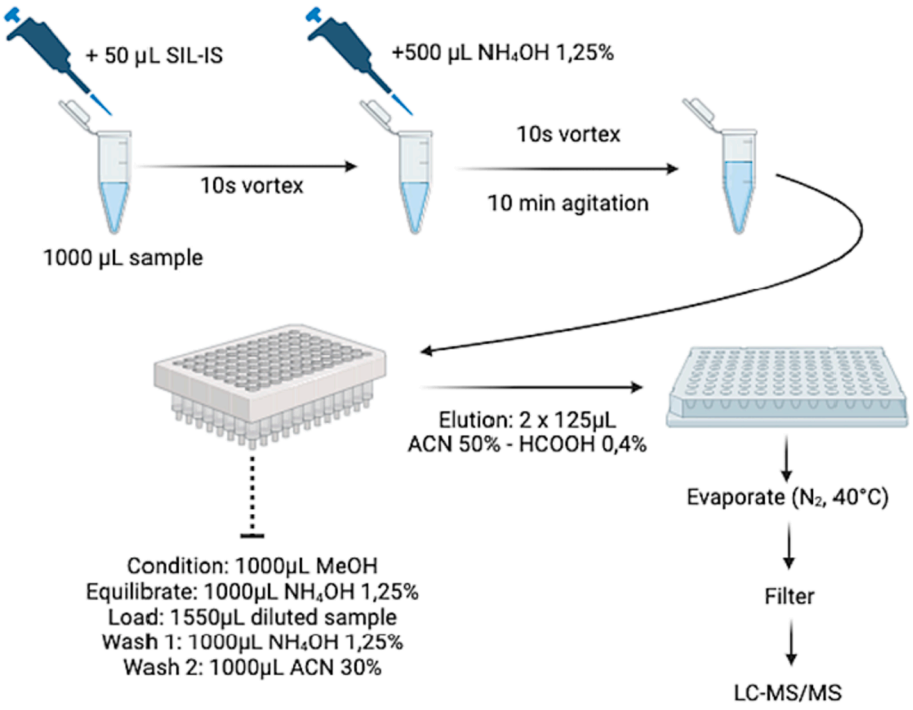

Fig. 2. Sample preparation scheme.

Table 2
MRM transitions, compound and source parameters.

ID	Q1 Mass	Q3 Mass	Time (msec)	DP (V)	EP (V)	CE (V)	CXP (V)
Ang (1–7)	450.4 $(MH)^{+2}$	392.9 $(y6)^{+2}$	150	55	10	17	10
Ang (1–7)	450.4 $(MH)^{+2}$	647.4 (b5)	75	55	10	20	10
IS Ang (1–7)	453.2	653.4	95	55	10	12	10
IS Ang (1–7)	453.2	395.9	50	55	10	19	10
Ang (1–8)	524.1 $(MH)^{+2}$	263.2 (y2)	110	55	10	26	10
Ang (1–8)	524.1 $(MH)^{+2}$	647.5 (b5)	75	55	10	29	10
IS Ang (1–8)	351.9	371.3	75	55	10	20	10
IS Ang (1–8)	527.6	263.1	50	55	10	25	10
Ang (1–9)	395.4 $(MH)^{+3}$	647.5 (b5)	110	55	10	16	10
Ang (1–9)	395.4 $(MH)^{+3}$	534.3 (b4)	75	55	10	22	10
IS Ang (1–9)	300.2	136.1	75	55	10	20	10
IS ang (1–9)	399.1	156.0	50	55	10	27	10
Ang (1–10)	433.1 $(MH)^{+3}$	619.3 (a5)	110	55	10	23	10
Ang (1–10)	433.1 $(MH)^{+3}$	647.4 (b5)	75	55	10	17	10
IS Ang (1–10)	437.4	660.5	75	55	10	20	10
IS Ang (1–10)	437.4	513.4	50	55	10	20	10

divided into 1 mL aliquots within 12 Protein LoBind Eppendorf tubes. Each aliquot was then subjected to the previously described processing steps.

The extracted samples were subsequently combined into a pooled sample, followed by an evaporation step until dryness was achieved. The resulting dried extracts were reconstituted using 100 µL of a solution containing H_2O, 5% ACN, and 0.4% FA. Subsequently, this reconstituted sample was injected into the mass spectrometer and analyzed using a method that encompassed the transitions corresponding to Ang (1–7), (1–8), (1–9), and (1–10), as well as the theoretical transitions of Ang (1–5), (2–10), (2–8), (2–7), (3–8), and (3–7).

2.5.2. Linearity

To assess the linearity of the method, a human plasma pool was spiked with a substantial concentration of Ang (1–10), Ang (1–8), Ang (1–7), and Ang (1–9). This spiked plasma was subsequently diluted using cow plasma that had also been diluted to varying percentages (20%, 40%, 60%, and 80%). Cow plasma was chosen as the blank matrix due to its close resemblance to human plasma while remaining almost angiotensin free. These samples were prepared in quintuplicate and subsequently subjected to analysis within the same analytical run.

2.5.3. Calibration curve

The construction of the 8-point calibration curve (ranging from 5 to 8000 pg/mL) adhered to the previously outlined procedure. The calibration curve was fitted using linear, quadratic, and cubic regression models, each subjected to a weighted $(1/x^2)$ approach. For the purpose of assessing linearity, the nonlinear fit was deemed acceptable when the difference between the nonlinear and linear fit outcomes was either less than or equal to 5%.

2.5.4. Precision and accuracy

Validation samples were prepared using cow plasma that had been diluted to 50% with PBS and 0.1% BSA (w/w). These samples were established at eight distinct concentration levels: 5 pg/mL (lower limit of quantification), 5.5 pg/mL, 10 pg/mL, 25 pg/mL, 50 pg/mL, 100 pg/mL, 1 ng/mL, and 5 ng/mL. The extraction process applied to these validation samples was consistent with the procedures used for regular samples. Each validation sample was subject to processing and analysis in four replicates over the course of five consecutive days.

Inter- and intra-run precisions, as well as accuracies, were evaluated using the single-nested analysis of variance (ANOVA) method, as

stipulated by CLSI EP05 guidelines. The levels of precision were expressed as coefficients of variation (CV %), while the accuracy was presented as mean recoveries (%).

2.5.5. Sample preparation recovery, ion suppression and matrix effects

Matrix factors were assessed through the addition of the same quantity of SIL-IS for each angiotensin to five extracted human plasma samples and a solvent extract (H_2O). The matrix factor was calculated by dividing the area under the curve (AUC) of the internal standard (IS) in the extracted human plasma sample by the AUC of the IS in the extracted solvent. The mean matrix factor was then expressed as a percentage (%).

Ion suppression was investigated using post-column infusion. A solution containing 100 ng/mL of native and SIL-IS Ang (1–7), (1–8), (1–9), and (1–10) was directly infused into the source. Concurrently, 50 μL of the human plasma extract was injected.

For the assessment of recoveries, a working solution of angiotensins standard at a concentration of 200 pg/mL was prepared in a solution comprising H_2O, 5% ACN, 0.4% FA, and 0.1% BSA. Subsequently, three samples, each consisting of 1 mL of diluted cow plasma, were spiked with 50 μL of the working solution. These samples were then subjected to the previously detailed processing procedure. Following reconstitution, 50 μL of the SIL-IS working solution was added to the extracts. As a reference, 100 μL of the extracted solvent was spiked with the equivalent mass of native and SIL-IS angiotensins as present in the diluted cow plasma. The calculation of absolute recoveries was achieved by dividing the mean area ratios of each analyte obtained in the diluted cow plasma samples by the corresponding area ratios obtained for the extracted solvent.

2.5.6. Carry over

Carry over was assessed by performing three consecutive injections of a standard solution containing angiotensins at a concentration of 10 ng/mL, followed by the subsequent injection of a solvent (composed of H_2O, 5% ACN, 0.4% FA, and 0.1% BSA).

2.5.7. Lower limit of the measurement interval (LLMI)

In accordance with the guidelines established by the CLSI EP05-A3, the Lower Limit of measurement interval (LLMI) was established as the lowest concentration that can be consistently and accurately detected while meeting the stipulated requirements for precision and accuracy. To ascertain this value, a serial dilution process was applied to a sample of diluted cow plasma until a concentration of 5 pg/mL was achieved. Subsequently, 40 replicates of this diluted cow plasma were subjected to analysis over a span of 10 days.

For the assessment of precision, the coefficient of variation (CV %) was employed, with the criterion set that it should not exceed 20%. In terms of trueness bias, it was ensured that it did not surpass 15%.

The Limit of Detection (LOD) was defined as values that fall below the established LLMI.

2.5.8. Stability

Sample stability was assessed by storing aliquots of a human plasma pool at 4 °C for 0–4 days, followed by transfer to −80 °C until processing and injection. While specific QC stability assessments were not conducted, it was assumed that the stability of QCs would parallel that of the samples, given their spiked plasma nature. Two pools, one with high (≈20 pg/mL) and the other with low (≈7 pg/mL) concentrations of angiotensin peptides, were created to explore the potential impact of concentration on sample stability. Neat solutions, comprised of identical standard solutions, underwent daily injections over 5 days while stored at 4 °C. Extracted samples, derived from two human plasma samples with varying concentrations, were processed, and the extracts were injected daily for five days, housed in the autosampler. Coefficients of variation were calculated by dividing the standard deviation by the mean.

2.6. Measurement uncertainty

In this validation process, four distinct types of uncertainties were computed: uncertainty of the bias, combined uncertainty, expanded uncertainty, and related uncertainty. The uncertainty of the bias was determined by dividing the standard deviation by the square root of 'n', where 'n' represents the number of replicates.

The combined uncertainty was calculated using the following formula:

$$\sqrt{\left(\frac{SD}{\sqrt{n}}\right)^2 + \left(\frac{average\ \ concentration - expected\ \ concentration}{\sqrt{3}}\right)^2} \quad (1)$$

The expanded uncertainty was evaluated by multiplying the calculated combined uncertainty by a factor 'K', where 'K' equals 2.

The related uncertainty was determined by dividing the combined uncertainty by the anticipated or expected concentration.

Considering the absence of available data on the biological variation of angiotensin peptides, a proactive decision was made to establish the Measurement Uncertainty (MU) goal as being below 15%.

2.7. Analysis of human plasma samples

Remnant samples were analysed utilizing the newly developed LC-MS/MS method. This dataset comprised 10 samples obtained from patients suffering from severe kidney failure, 10 samples originating from patients with high blood pressure, and an additional 10 samples drawn from healthy individuals. Remnant samples were specifically chosen from patients exhibiting a glomerular filtration rate below 20 for individuals with chronic kidney disease. For patients diagnosed with high blood pressure, remnant samples were selected after determination of renin activity and aldosterone levels for routine needs. Selected patients were treated with pressure reducing medicines. All samples were collected in EDTA tubes and immediately stored at −20 °C after the routine analysis. As for healthy individuals, we obtained plasma samples from 10 willing and healthy volunteers. Uniformly, all samples underwent processing and analysis on the same day, within a single analytical run.

3. Results

3.1. Selectivity

Our liquid chromatography method effectively resolved all the diverse angiotensins known to be physiologically present in humans, including Ang (1–5), (2–10), (2–8), (2–7), (3–8), (3–7), (1–7), (1–8), (1–9), and (1–10). This successful resolution is visually depicted in Fig. 3.

While there are no instances of co-elution, we have thoroughly examined for potential transition interferences. Notably, none of the unmeasured angiotensin peptides exhibited peaks that coincide with the retention times of Ang (1–7), (1–8), (1–9), and (1–10). However, it is worth noting that transition m/z 399 > m/z 156 (Ang (1–9) IS) exhibited a peak corresponding to the retention time of Ang (1–5), and transition m/z 300 > m/z 136 (Ang (1–9) IS) demonstrated a peak at the retention time of Ang (2–10).

3.2. Linearity

Examinations of the diluted samples showed anticipated proportional values, affirming a direct correspondence between the real concentrations of the samples and the recorded outcomes, as illustrated in the Table 3.

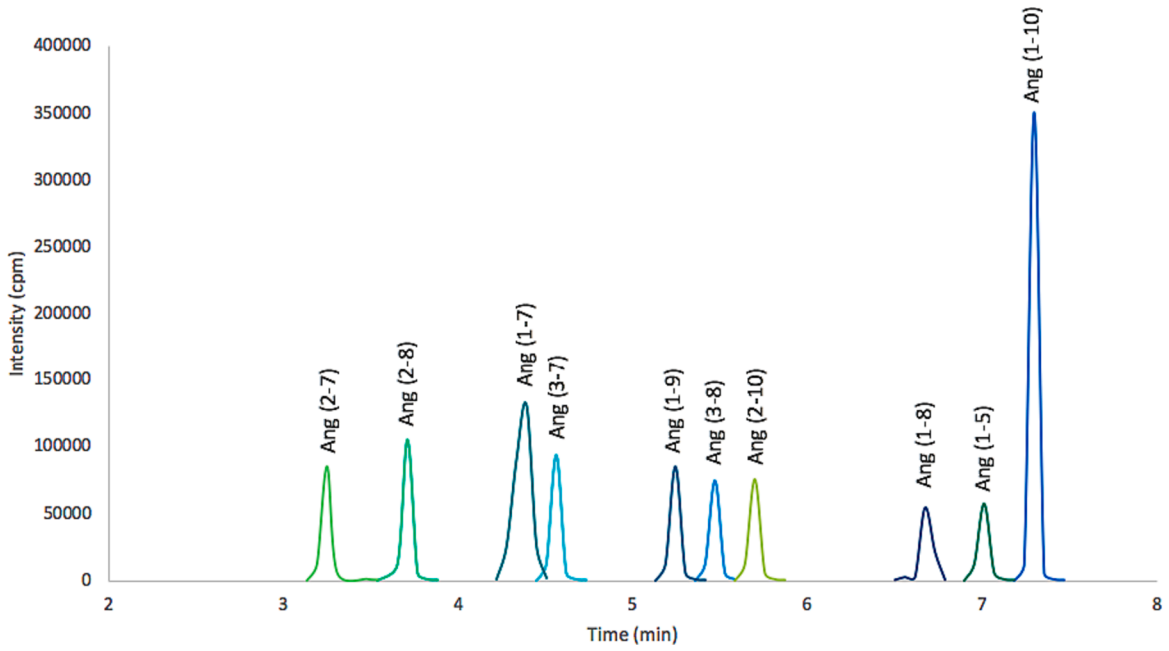

Fig. 3. Schematic chromatogram displaying the retention time obtained for all of the angiotensin peptides.

Table 3
Values obtained for the linearity study.

	Calculated Concentration				Expected Concentration				Accuracy			
	Ang (1–7)	Ang (1–9)	Ang (1–8)	Ang (1–10)	Ang (1–7)	Ang (1–9)	Ang (1–8)	Ang (1–10)	Ang (1–7)	Ang (1–9)	Ang (1–8)	Ang (1–10)
20%	1874,0	1199,0	1484,2	1436,6	1914,7	1086,7	1384,7	1253,3	98%	110%	107%	115%
40%	3695,6	2281,3	2759,3	2565,9	3829,5	2173,5	2769,4	2506,5	97%	105%	100%	102%
60%	5696,4	3379,2	4136,4	3742,9	5744,2	3260,2	4154,1	3759,8	99%	104%	100%	100%
80%	7903,0	4490,5	5547,6	5069,5	7659,0	4347,0	5538,8	5013,1	103%	103%	100%	101%

3.3. Calibration curve

Various regression models were applied to the calibration curve, with the linear model utilizing weighted ($1/x^2$) being chosen as the preferred option. The linearity of the curve was regarded as acceptable, given that the difference between the nonlinear and linear fit outcomes was below the threshold of 5%.

3.4. Precision and accuracy

During the validation process, the accuracy and precision for intra- and inter-day analyses were established across eight QC levels spanning from 5 pg/mL to 8000 pg/mL.

The accuracy, represented by recoveries, ranged from 93% to 107% for Ang (1–10), from 91% to 109% for Ang (1–9), from 91% to 109% for Ang (1–7), and from 92% to 109% for Ang (1–8).

For intra-day analyses, the coefficient of variation (CV %) ranged from 1% to 13% for Ang 1–10, from 1% to 13% for Ang (1–9), from 1% to 11% for Ang (1–7), and from 1% to 10% for Ang (1–8). In terms of inter-day analyses, the CV spanned from 3% to 7% for Ang (1–10), from 3% to 6% for Ang (1–9), from 4% to 8% for Ang (1–7), and from 2% to 6% for Ang 1–8. All inter and intra-day CVs and absolute recoveries obtained during the validation are presented in the supplemental Tables 1–4.

3.5. Sample preparation recovery, ion suppression and matrix effects

The absolute sample recoveries ranged from 58% to 62%. The calculated matrix effect spanned from 91.6% to 97.8%.

Peaks were observed with retention times of 4.6 min, 5.4 min, 6.8 min, and 7.4 min, corresponding to Ang (1–7), (1–8), (1–9), and (1–10) respectively. Importantly, there were no notable signs of ion suppression detected during these specific retention times.

3.6. Carry over

No significative deviation from background noise was observed subsequent to a 10 ng/mL injection. Consequently, carry over was deemed negligible, particularly considering the LLMI.

3.7. Low limit of the measurement interval (LLMI)

The quality control (QC) level set at 5 pg/mL exhibited an average intraday CV of 7% for Ang (1–10) and (1–7), and a CV of 5% for Ang (1–9) and (1–8). Accordingly, this level was designated as the LLMI. Fig. 4 visually portrays the response corresponding to the LLMI.

3.8. Measurement uncertainty

The calculated values for relative uncertainties ranged between 1.81% and 6.93% and were thus consistently under 15%.

3.9. Stability

Regarding the stability of plasma samples stored for 1 to 5 days at 4 °C, all CVs remained below 15%. Notably, a minor decline was observed throughout the days for all angiotensin peptides, especially at low concentrations. For the extracted samples, those at high

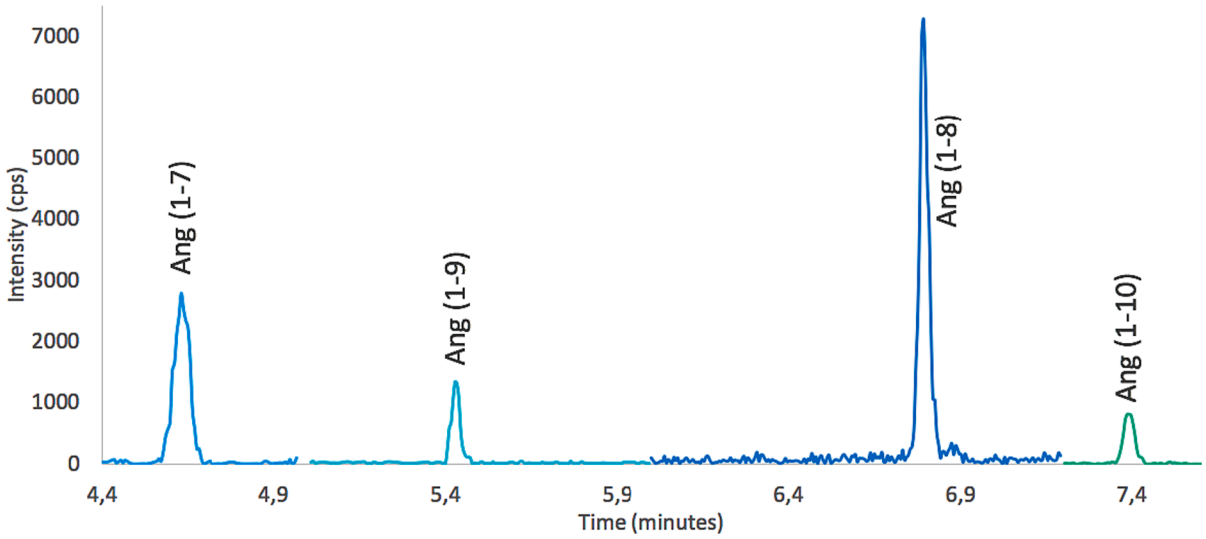

Fig. 4. Chromatogram obtained for validation samples at 5 pg/mL.

concentrations remained stable (CVs < 15%) throughout the five days, while samples at low concentrations were stable only during the first two days spent in the autosampler. Clean solutions remained stable for 5 days when stored at 4 °C and homogenized before injection.

3.10. Analysis of human plasma samples

In all analysed samples, we successfully observed the presence of all angiotensin peptides. Nevertheless, in the case of Ang (1–9), more than 80% of the samples were detected below LLMI, making their accurate quantification unfeasible.

For Ang (1–7), (1–8), and (1–10), accurate concentrations were successfully calculated for almost each sample. Notably, patients with chronic kidney disease exhibited higher levels of angiotensin peptides, which can be attributed to their decreased clearance due to impaired renal function, in contrast to the other groups. We identified outliers with significantly elevated angiotensin peptide levels in both the chronic kidney disease group (n = 2) and the high blood pressure group (n = 2). These outcomes are presented in the box plot illustrated in Fig. 5 and Table 4.

Furthermore, we observed matrix effects in samples from patients with chronic kidney disease. Specifically, internal standard peak heights decreased by 20% in these patients. Nevertheless, the peaks of native angiotensin peptides remained distinctly visible.

Considering our method primary aim of quantitating angiotensin peptides associated with the RAAS and alternative RAAS cascade, we are confident that we have achieved our objectives from an analytical perspective.

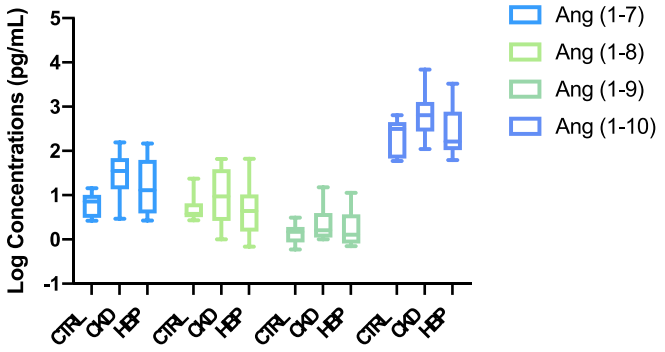

Fig. 5. Box plot representing the levels calculated for Ang (1–7), (1–8), (1–9) and (1–10) in the three groups (controls, high blood pressure patients (HBP) and chronic kidney disease patients (CKD).

4. Discussion

In this study, we have successfully developed a highly sensitive LC-MS/MS method that allows for precise quantitation of four pivotal angiotensin peptides: Ang (1–7), (1–8), (1–9), and (1–10). Our method was designed to facilitate comprehensive investigations into the diagnostic and prognostic potential for patients experiencing symptoms associated with high blood pressure and other chronic diseases. The validation process has emphasized the method's exceptional specificity and sensitivity. Notably, our method capacity to achieve chromatographic separation for all angiotensin peptides grants it a distinct advantage over immunoassays, which are often susceptible to cross-reactivity issues, particularly when dealing with peptides sharing highly similar sequences.

Our strict adherence to the CLSI guidelines further confirms the suitability of our method for the analysis of patient samples. This affirmation is reinforced by our analysis and quantitation of plasma samples, encompassing both healthy individuals and patients affected by a range of chronic diseases. Despite the slight matrix effect observed in patients with chronic kidney diseases, we were able to analyse and quantitate the plasma samples without necessitating additional sample preparation steps.

Elevated levels of Ang (1–10) were detected in all subject groups. This phenomenon may be attributed to the cleavage of angiotensinogen by renin, resulting in the production of Ang (1–10), a process not inhibited by EDTA. Notably, the generation of Ang (1–10) can occur at room temperature, whereas the formation of other angiotensin peptides is impeded by the presence of EDTA in the plasma. Consequently, our assessment of Ang (1–10) may not accurately represent the true endogenous levels. To mitigate this limitation, the addition of renin inhibitors to the samples before thawing is recommended to prevent ex-vivo generation of Ang (1–10) [20].

This matrix effect may be attributed to the elevated peptide levels present in patients with chronic kidney disease. These patients exhibit diminished filtration rates of peptides, consequently leading to higher peptide concentrations in their blood. This explains the higher results observed in patients with chronic kidney diseases, aligning well with the aforementioned previous explanation.

Our method ability to overcome such matrix effects and generate reliable quantitation underscores its robustness and applicability, thus positioning it as a valuable tool in the assessment of angiotensin peptides

Table 4
Results obtained for the thirty patients.

	CKD PATIENTS				HBP PATIENTS				CONTROLS			
(pg/mL)	ANG 1–8	ANG 1–10	ANG 1–9	ANG 1–7	ANG 1–8	ANG 1–10	ANG 1–9	ANG 1–7	ANG 1–8	ANG 1–10	ANG 1–9	ANG 1–7
PATIENT 1	13,7	832,3	<5	39,0	9	337,2	<5	11,7	<5	277,4	<5	5,5
PATIENT 2	<5	129	<5	<5	<5	137,6	<5	11,0	<5	359,1	<5	6,1
PATIENT 3	6,3	490,5	<5	21,3	<5	584,5	<5	147,0	<5	381,9	<5	9,4
PATIENT 4	15,4	921,2	<5	33,0	<5	90,7	<5	14,5	<5	59,1	<5	<5
PATIENT 5	45,1	2229,1	5,5	115,0	66,3	3275,4	11,33	53,6	<5	66,9	<5	<5
PATIENT 6	3,9	436,8	<5	21,7	<5	158.0	<5	<5	<5	67,4	<5	<5
PATIENT 7	<5	110,2	<5	<5	5,8	168,8	<5	<5	5,9	439,3	<5	10,7
PATIENT 8	<5	351,8	<5	58,0	<5	109,2	<5	15,3	<5	178,7	<5	8,5
PATIENT 9	36,5	1071,4	<5	38,1	15,9	1746,9	<5	102,9	9,1	479,3	<5	10,1
PATIENT 10	65,9	6849,6	15,1	155,7	<5	62,3	<5	<5	23,4	645,6	<5	14,4

across diverse patient populations.

5. Conclusion

In summary, our LC-MS/MS method has demonstrated its capacity for precise quantitation of four significant angiotensin peptides: Ang (1–7), (1–8), (1–9), and (1–10). Through the quantification of these peptides, we can effectively evaluate the activity of ACE and ACE2, which holds the potential to serve as valuable diagnostic and prognostic markers across a spectrum of diseases.

CRediT authorship contribution statement

Justine Demeuse: Methodology, Validation, Investigation, Writing – original draft, Visualization. **Loreen Huyghebaert:** Methodology, Validation, Investigation, Writing – original draft, Visualization. **William Determe:** Investigation, Writing – review & editing, Validation. **Matthieu Schoumacher:** Formal analysis, Software, Writing – review & editing. **Elodie Grifnée:** Methodology, Writing – review & editing. **Philippe Massonnet:** Writing – review & editing. **Thomas Dubrowski:** Writing – review & editing. **Marwa Rechchad:** Investigation. **Jordi Farre Segura:** Formal analysis. **Stéphanie Peeters:** Writing – review & editing. **Etienne Cavalier:** Conceptualization, Supervision, Project administration, Funding acquisition, Writing – review & editing. **Caroline Le Goff:** Project administration, Supervision, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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During the preparation of this work the author(s) used ChatGPT/OpenAI in order to improve language. After using this tool/service, the authors reviewed and edited the content as needed and take full responsibility for the content of the publication.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jchromb.2023.123943.

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