



Quantification of endogenous Angiotensin 1-10, 1-9, 1-8, 1-7, and 1-5 in human plasma using micro-UHPLC-MS/MS: Outlining the importance of the pre-analytics for reliable results

Jonathan Maurer^a, Anke de Groot^{a,1}, Léon Martin^{a,1}, Eric Grouzmann^a, Grégoire Wuerzner^b, Philippe J. Eugster^{a,*}

^a Service of Clinical Pharmacology, Lausanne University Hospital and University of Lausanne, Lausanne, Switzerland

^b Service of Hypertension and Nephrology, Lausanne University Hospital and University of Lausanne, Lausanne, Switzerland

ARTICLE INFO

Keywords:

Hypertension
Angiotensin
ACE
Preanalytical stability
Mass spectrometry

ABSTRACT

Angiotensin peptides (ANGs) play a central role in the renin–angiotensin–aldosterone system, rendering them interesting biomarkers associated with hypertension. Precise quantification of circulating ANGs holds the potential to assess the activity of angiotensin-converting enzyme (ACE), a key protease targeted by widely prescribed drugs, namely ACE inhibitors. This ability could pave the way for personalised medicine, offering insights into the prescription of inhibitors targeting either the proteases or the receptors within the system. Despite recent developments in liquid chromatography–mass spectrometry (LC-MS) methods for measuring circulating ANG concentrations, comprehensive stability studies of ANGs in human plasma are absent in the literature, raising concerns about the reliability of measured concentrations and their link to clinical conditions. To address this critical gap, we conducted an exhaustive evaluation of the pre-analytical stability of ANG1–10, ANG1–9, ANG1–8, ANG1–7, and ANG1–5. By employing surfactants to mitigate non-specific adsorption and a dedicated mix of protease inhibitors to limit protease activity, we established an MS-based assay for these five peptides. We used this method to quantify circulating concentrations of ANGs in the plasma of 11 healthy donors and 3 patients under kidney dialysis. Our findings revealed that ANG1–10 and ANG1–8 circulate at concentrations ranging from 1 to 10 pM in healthy subjects and exhibit a high degree of correlation. Notably, ANG1–9, ANG1–7, and ANG1–5 were undetectable in any of the 14 patients, despite a sub-picomolar limit of detection. This strikingly contrasts with the reference concentrations reported in the literature, which typically fall within the picomolar range. In light of these discrepancies, we strongly advocate for rigorous pre-analytical considerations and comprehensive stability studies to ensure reliable results. We emphasise the pivotal role of heightened pre-analytical awareness within the clinical chemistry community, and we hope for continued growth in this critical area.

1. Introduction

The renin–angiotensin–aldosterone system (RAAS) is a complex but fundamental system in the human body; it comprises hormones, enzymes, and receptors that control the blood pressure [1,2]. Starting from angiotensinogen, a pro-peptide synthesised by the liver, the process involves renin, a proteolytic enzyme from the juxtaglomerular apparatus [1,2]. Renin production is triggered by reduced blood pressure sensed by baroreceptors, ionic imbalance detected by macula densa cells, and

sympathetic nerve system stimulation via the β_1 receptor in response to low blood pressure. Renin converts liver-produced angiotensinogen to angiotensin (ANG) I [1,2]. ANG I, or ANG1–10, is the precursor for various ANGs. The octapeptide ANG II, or ANG1–8 is formed by cleaving two amino acids from ANG1–10 by angiotensin-converting enzyme (ACE). It acts on two G-protein coupled receptors: angiotensin II receptor type 1 (AT1R), which increases blood pressure through vasoconstriction, aldosterone release, and kidney sodium and water reabsorption, and angiotensin II receptor type 2 (AT2R), which

* Correspondence to: Avenue Pierre-Decker 5, Lausanne 1011, Switzerland.

E-mail address: philippe.eugster@chuv.ch (P.J. Eugster).

¹ These authors contributed equally

<https://doi.org/10.1016/j.jpba.2024.116101>

Received 12 January 2024; Received in revised form 26 February 2024; Accepted 7 March 2024

Available online 9 March 2024

0731-7085/© 2024 The Author(s). Published by Elsevier B.V. This is an open access article under the CC BY license (<http://creativecommons.org/licenses/by/4.0/>).

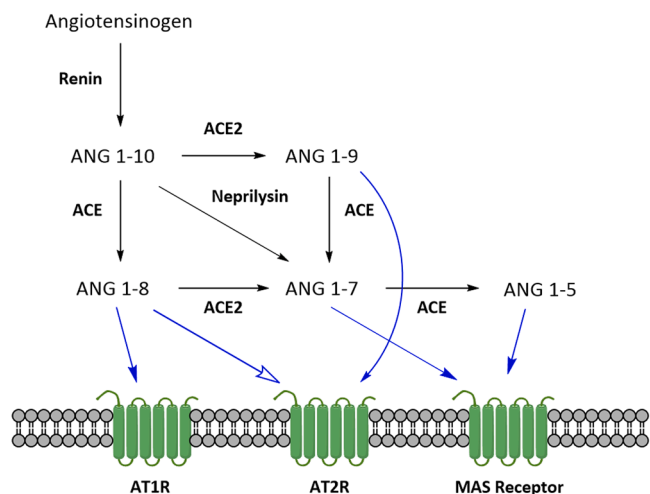


Fig. 1. : The RAAS pathway. Angiotensinogen is cleaved by renin to form ANG1-10. ANG1-10 is further metabolised to ANG1-9, ANG1-8, or ANG1-7, by ACE2, ACE, and neprilysin, respectively. ANG1-8 via ACE2 and ANG1-9 via ACE can also be metabolised to ANG1-7. ACE forms ANG1-7 from ANG1-5. This figure was made using the ChemDraw application.

promotes vasodilation and natriuresis and inhibits inflammation and fibrosis. The nonapeptide ANG1-9 is formed by angiotensin-converting enzyme 2 (ACE2)-mediated cleavage of one C terminal amino acid from ANG1-10. ANG1-9 also acts on AT2R. It mediates anti-hypertrophic effects, vasoprotective actions, and counterbalances the activity of ACE and ANG1-8 [3]. ANG1-7 is derived from neprilysin cleavage of ANG1-10, ACE cleavage of ANG1-9, or ACE2 cleavage of ANG1-8. ANG1-7 acts on the MAS receptor and to a lesser extent on AT2R, exhibiting similar effects as ANG1-9 [3,4]. ANG1-7 is further metabolised to the pentapeptide ANG1-5 by ACE [5]. ANG1-5 acts on the MAS receptor, showing cardioprotective effects like ANG1-9 and ANG1-7. Although more ANG peptides and enzymes are involved, we focus on the five aforementioned ANGs: ANG1-10, ANG1-9, ANG1-8, ANG1-7, and ANG1-5 [6]. Fig. 1 summarises the metabolisms of these ANGs.

The RAAS plays an important role in many diseases, particularly hypertension. RAAS activation increases blood pressure through vasoconstriction, increased vascular resistance, and an expanded blood volume [7-9]. However, inappropriate RAAS activation has detrimental effects, contributing to cardiovascular diseases such as stroke, coronary heart disease, heart failure, major cardiovascular events, and atherosclerosis [7,10-12]. ANG1-8, in particular, stimulates vascular and cardiac growth, proliferation, endothelial dysfunction, inflammation, and cellular damage due to increased reactive oxygen species accumulation [10-12]. RAAS activation also promotes inflammation, hypertension, proteinuria, and fibrosis, leading to kidney damage [7,10]. As a result, any imbalance in this system can result in chronic and acute diseases.

Therefore, a robust and specific method for quantifying ANGs is essential to understand their roles in physiological and pathological conditions. Additionally, ANG concentrations can be used to calculate ACE and ACE2 activity *in vivo*. These parameters enhance our understanding of RAAS-related diseases and therapies and can also help categorise patients based on the hypertension type for personalised medicine. For example, identifying which patients respond better to angiotensin II receptor blockers (ARBs), angiotensin-converting enzyme inhibitors (ACEi), or direct renin inhibitors (aliskiren) represents a significant step forward in hypertension treatment.

Previously, the quantification of ANG peptides was commonly carried out by immunoassays (IAs) [13]. However, because the ANG peptides share the same structure and differ only by the truncation of one or two amino acids, IAs may introduce bias through cross-reactivity [14].

Alternative techniques, such as capillary electrophoresis combined with electrochemical detection or combined with ultraviolet (UV) detection, lack sufficient sensitivity to determine endogenous ANG concentrations [15]. Moreover, these techniques are not adequate for the separation of peptides in a complex matrix such as plasma. They must be coupled with more selective tools such as mass spectrometry. Fluorescence detection, while feasible, is more expensive and time-consuming due to derivatization of the terminal amine group [16]. Recently, liquid chromatography combined with mass spectrometry (LC-MS) has emerged as the gold standard for quantifying ANGs [17-21]. Compared with IAs, MS detection offers higher specificity, the ability to quantify multiple peptides simultaneously, and the use of an internal standard (IS) to correct analytical variations [22]. However, LC-MS is not perfect as it is a very expensive method, with a need of high expertise and regular support for the maintenance of the instruments.

Beyond analytical considerations, pre-analytical conditions must be carefully evaluated to prevent losses due to the specific properties of these peptides. ANGs tend to adsorb onto the surface of materials during storage and sample preparation, significantly affecting quantitative analysis results [22,23]. Additionally, ANGs are continuously cleaved by proteases of the RAAS system (Fig. 1). Therefore, to measure circulating concentrations of ANGs accurately, it is crucial to prevent *ex vivo* degradation after collection. To this end, protease inhibitors are added to the collection tubes before sample collection and preparation, to stop any further generation or breakdown of ANGs. Furthermore, ANGs circulate in the human plasma in the picomolar range and depend on many individual factors including circadian rhythm (the concentrations are 2-3 times higher in the morning than during the rest of day), physical activity, sex, sodium consumption, age, medication, stress, and blood collection position [24-27]. As a result, reference intervals must be based on samples with harmonised collection conditions.

This article presents the development and validation of an assay to simultaneously quantify five endogenous ANGs in the human plasma using ultra-high-performance liquid chromatography coupled to tandem mass spectrometry (UHPLC-MS/MS), with a special focus on ANG stability from sampling to analysis using protease inhibitors and surfactants. Subsequently, the validated method was used on plasma samples from healthy donors to estimate circulating concentrations of ANGs.

2. Material and methods

2.1. Chemical and reagents

ULC-MS-grade water, acetonitrile (ACN), isopropanol, methanol (MeOH) absolute, and formic acid (FA) 99% were purchased from Biosolve (France). Trifluoroacetic acid (TFA) 99+% and dimethyl sulphoxide (DMSO) were obtained from Thermo Fisher scientific (USA). Ammonia 25% was procured from Suprapur (Germany). 2,2,2-Trifluoroethanol 99+% was from Alfa Aesar (USA). *n*-Nonyl β -D glucopyranoside (NG) was purchased from Neo Biotech (France). Phosphate-buffered saline was obtained from Bichsel (Switzerland). Bovine serum albumin, aliskiren, 2,2'-bipyridyl, and ethylenediaminetetraacetic acid (EDTA) were purchased from Sigma-Aldrich (USA). Captopril was obtained from Labforce AG (Switzerland). Angiotensin peptides and their ISs were purchased from GeneCust (France). Collection tubes were from Eppendorf SE (Germany) and Minisorp tubes from Nunc-Immuno (Thermo Scientific, USA). Blood was collected in S-Monovette 4.9 mL K3E tubes containing EDTA that were purchased from Sarstedt AG & Co. KG (Germany).

IS were prepared with ^{13}C , ^{15}N labelling on Val and Ile (+ 13 Da) for ANG 1-10, and with ^{13}C , ^{15}N labelling on residue Ile (+ 7 Da) for ANG1-9, ANG1-8, ANG1-7, and ANG1-5.

Uptilates 96-well protein crash 2 mL 0.20 μm filter plates were obtained from Interchim (France). The Acquity UPLC M-Class system, the Xevo TQ-S mass spectrometer, the CSH C18 IonKey/MS with particle size of 1.7 μm and dimensions of 150 μm \times 50 mm, the nanoEase M/Z

Symmetry C18 with particle size of 5 μm and dimensions of 300 μm x 5 μm , square 2 mL 96-well sample collection plates, Acquity UPLC 700 μL round 96-well sample collection plates, the positive pressure-96 processor, and Oasis hydrophilic-lipophilic balance (HLB) 96-well plates 30 μm (5 mg) were purchased from Waters (Milford, MA, USA). The ROTINA 420 R and MIKRO 220 R centrifuges and Thermomixer were obtained from Hettich (France). The STR4 rotator was obtained from Stuart (UK). The TurboVap 96 evaporator was obtained from Biotage (Sweden).

The Masslynx V4.2 and Targetlynx XS software from Waters were used to produce and analyse the chromatograms. Plots were made with GraphPad Prism 9.5.1. (GraphPad Software, USA) Differences between two means was evaluated using Student t-test with a significance threshold set at $p < 0.05$.

2.2. Patients and sample collection

Samples were collected from 11 healthy volunteers and 3 patients with kidney failure recruited at the Service de Néphrologie et d'Hypertension of the CHUV, Lausanne. An Additional anonymized blood sample of 4.5 mL was taken from participants of an observational study after written consent was obtained. The observational study had received ethical clearance from the local ethic committee. No demographic data or identification data were sent to the laboratory.

Before collection, each patient laid down for 15 minutes. Blood was collected in 4 $^{\circ}\text{C}$ pre-chilled 4.5 mL EDTA tubes containing an in-house optimised protease inhibitor cocktail composed of captopril, EDTA, aliskiren, and 2,2'-bipyridyl at a final blood concentration of 0.04 mg/mL, 40 mM, 0.04 mM, and 1.5 mg/mL, respectively. The mix was prepared in a ready-to-use lyophilised batch and resolubilised in 250 μL of water before addition to the 4.5 mL collection tubes. After collection, the tubes were kept on ice and centrifuged within 30 minutes in a 4 $^{\circ}\text{C}$ pre-chilled centrifuge for 10 minutes at 2500 RCF. The plasma was then aliquoted on ice (500 μL per aliquot) in 1.5 mL Eppendorf tubes and stored at -80°C until analysis.

2.3. Calibration curve and quality control (QC) samples

Lyophilisates of 1 mg neat ANGs were solubilised, and the peptide content and the purity were considered to obtain a net ANG concentration of 1 mM for each solution. Calibrants were prepared in a surrogate matrix of pooled plasma supplemented with a solution containing protease inhibitors and diluted 10 times in water. The 1 mM solutions were mixed to a final concentration of 30 nM, for ANG1-10 and ANG1-8, and 150 nM, for ANG1-9, ANG1-7, and ANG1-5. The solution was aliquoted in 5 mL Minisorp tubes and lyophilised to increase the peptide stability. When needed, the ready-to-use batches were solubilised in a volume of FA 0.1%-NG 0.1% to reach the original concentrations. Eight calibrants were prepared by spiking 50-0.5 pM of ANG1-10 and ANG1-8, and 250-2.5 pM of ANG1-9, ANG1-7, and ANG1-5 (Table S1). The actual ANG concentrations in the calibrants is the sum of the spiked ANGs plus the endogenous concentration measured for each new batch by using a linear curve made of triplicates of the 50 pM point with 1/x fit weighting and a curve forced by zero.

QC samples were prepared independently of the calibrants in a plasma matrix made by pooling plasma samples together and supplemented with protease inhibitors. Again, 1 mM solutions were mixed to a final concentration of 20, 12.5, and 0 nM for ANG1-10 and ANG1-8, and 100, 62.5, and 7.5 nM for ANG1-9, ANG1-7, and ANG1-5, depending on the QC concentration (High, Medium, and Low). The solution was aliquoted in 5 mL Minisorp tubes, lyophilised, and solubilised in a volume of FA 0.1%-NG 0.1% to reach the original concentrations when needed. Three levels of QC samples were prepared, depending on the lower limit of quantification (LLOQ) of the corresponding peptide: the QC low, medium, and high were spiked with 15, 125, and 200 pM of ANG1-9, ANG1-7, and ANG1-5, and 0, 25, and 40 pM of ANG1-10 and

ANG1-8, respectively. The spiked concentrations were chosen according to the expected concentrations found in the literature. The actual concentrations are the sum of the endogenous concentrations in the pooled plasma matrix that fluctuates from batch to batch, and the spiked concentrations (Table S2).

2.4. Sample preparation

An IS solution containing 4 nM of $^{13}\text{C},^{15}\text{N}$ -labeled ANG1-10, ANG1-9, ANG1-8, ANG1-7, and ANG1-5 in a solution containing FA 0.1%-NG 0.1% was prepared. Plasma samples were thawed for 10 minutes at room temperature in front of a fan and centrifuged at 2500 RPM for 10 minutes. Protein precipitation was performed in 2 mL Eppendorf tubes containing 900 μL of ACN, 10 μL of the IS solution, in which 450 μL of the sample were added; the tube was immediately vortexed for 10 seconds. Each sample was incubated for 20 minutes on a rotator at 60 RPM and then centrifuged at 21,250 RCF for 10 minutes. Supernatants were filtered on a protein crash that was previously washed two times with 100 μL of TFA 1% in MeOH. Filtrates were evaporated to dryness at 45 $^{\circ}\text{C}$ under an increasing nitrogen flow. After evaporation (approximately 3 hours), they were reconstituted in 450 μL of FA 1%-NG 0.1%, centrifuged at 1000 RCF for 1 minute, shaken at 1200 RPM for 5 minutes, and centrifuged at 2500 RCF for 10 minutes. The supernatants were loaded on a solid-phase extraction (SPE) plate preconditioned with 500 μL of MeOH and equilibrated with 500 μL of water. The samples were washed with 500 μL of MeOH 5% and eluted in a 700 μL plate with FA 5%-NG 0.1% in MeOH. The plate was then centrifuged at 1000 RCF for 1 minute and the eluates evaporated to dryness at 45 $^{\circ}\text{C}$ under an increasing nitrogen flow. Before injection in the LC-MS apparatus, the samples were reconstituted in 40 μL of FA 0.1%-NG 0.1%-MeOH 10%, centrifuged at 1000 RCF for 1 minute, shaken at 1200 RPM for 5 minutes, and centrifuged at 2500 RCF for 10 minutes.

2.5. Micro-UHPLC-MS/MS analyses

Separation was performed on a Waters Acquity UPLC M-class system configured for trap and back-flush elution with an auxiliary pump (ASM), a trap unit (TVM), and an IonKey device. Mobile phase A was composed of DMSO 1% and FA 0.05% in water, while mobile phase B was composed of DMSO 1% and FA 0.05% in ACN. The auxiliary solvent was composed of MeOH 2% and FA 0.1% in water. The weak wash was composed of FA 0.05% in water and the strong wash was water 25%, ACN 25%, MeOH 25%, and isopropyl alcohol 25%. The seal wash was composed of ACN 5% in water. The auxiliary solvent was delivered at a flow rate of 20 $\mu\text{L}/\text{minute}$. The separation was performed at a flow rate of 3 $\mu\text{L}/\text{minute}$, with a linear gradient from 2% to 95% B over 7 minutes. This composition was maintained for 4 minutes and then decreased to 2% B in 0.5 minutes and maintained at this composition for 3.5 minutes. The total runtime was 15 minutes. The injection volume was 30 μL on a 20 μL loop, using full loop mode with an overflow factor of 1.5. The transitions used to quantify and qualify the analytes are shown in Table 3S.

2.6. Method validation

The method was validated according to the current bioanalytical method validation guidelines of the US Food and Drug Administration (FDA) [28], the European Medicines Agency (EMA) [29], the Clinical & Laboratory Standards Institute (CLSI) [30], and peptide working recommendations [31].

Linearity was assessed via r^2 , the slope, and the y-intercept measured on the back-calculated values of calibrants from three independent runs. Intra-assay precision was determined by analysing quintuplicates of the QC samples. Inter-assay precision was evaluated on three separate analyses of quintuplicates of the QC samples. Accuracy is not reported

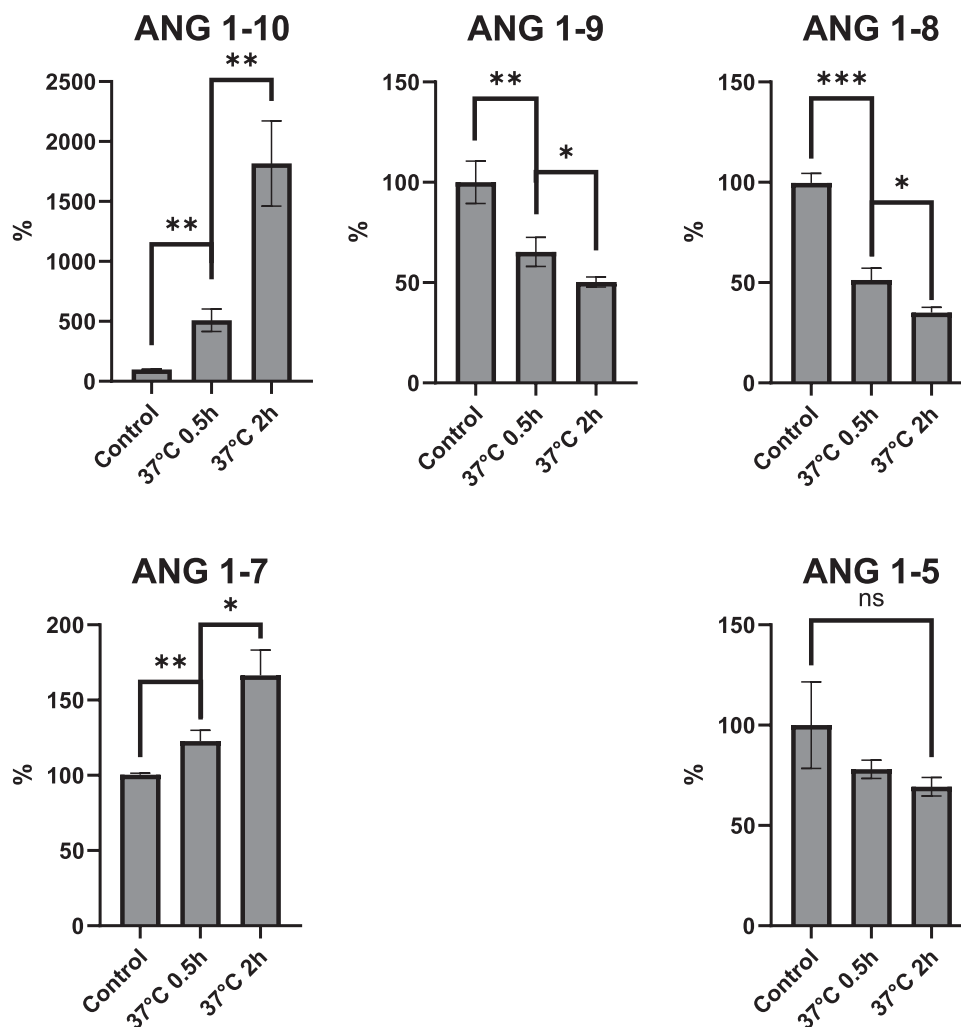


Fig. 2. : Relative ANG concentrations found after incubation at 37 °C for 0.5 and 2 hours in plasma spiked with 1000 pM of each ANG. The control is the same plasma but stored at –80 °C. ns $p > 0.05$; * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$.

here, as no perfectly blank matrix was available, thus lowering the interest of bias calculation, particularly for low concentrations. Recovery and matrix effects were studied as detailed by Matuszewski et al. [32] on three plasma samples from volunteers spiked with 100 pM ANG. The matrix effect was also evaluated in haemolysed plasma. The LLOQ was determined by analysing quintuplicates of plasma samples spiked with known concentrations of peptides. For ANG1–10 and ANG1–8, plasma was diluted 10 times in water to reduce the endogenous ANG concentration below the LLOQ. The LLOQ was determined as the lowest concentration providing a coefficient of variation (CV) < 20%. Carryover was estimated by analysing the lowest calibrant, the highest calibrant, and then a blank solution (FA 0.1%–NG 0.1%–MeOH 10%). This was repeated three times. Carryover is expressed as the ratio of the area measured for the blank on the area measured for the lowest calibrant. Carryover was considered acceptable if < 10%.

2.7. Stability study

The peptide stability was studied to ensure ANG stability in each step of the assay.

The freeze and thaw stability of ANG in plasma was evaluated by using triplicates of native plasma containing protease inhibitors and spiked with ANG at a concentration equivalent to QC high. The test was performed for three cycles, with > 24 hours at –80 °C and > 10 minutes at +22 °C. All samples were extracted together, and concentrations were

compared with reference samples stored at –80 °C.

Short-term stability was studied for all critical steps of the analysis, from blood collection to storage at –80 °C. To evaluate the need of inhibitors to stop the protease activity, a first stability study was conducted, by adding 1000 pM of every ANG peptide to EDTA-collected plasma. The plasma was then stored at –80 °C or incubated at 37 °C for 0.5 and 2 hours. For the stability in blood collection tube, blood was collected in a tube supplemented with the mix of protease inhibitors solubilised in water, directly centrifuged, and then spiked with 1000 pM of ANG, and it was compared to a similar tube that had been spiked before the blood collection and incubated for 30 minutes on ice before the centrifugation. To mimic the extraction, stability was tested by incubating the sample for 1 hour at room temperature and for 1 hour on ice, using the plasma of six donors supplemented with the protease inhibitor cocktail and spiked with a concentration equivalent to QC high. This approach allowed us to evaluate the inter-variability of enzymatic activity. Finally, to assess the stability of leftover samples in a fridge, stability was tested after storing a plasma sample spiked with 1000 pM of ANG for 24 hours at 4 °C.

Extracted sample stability was evaluated by comparing QC samples extracted and directly frozen in their dried form with QC samples extracted, solubilised, and incubated for 72 hours in the autosampler set at 10 °C.

Long-term stability of ANG in plasma was monitored for QC high, medium and low samples prepared in plasma, stored at –80 °C, and

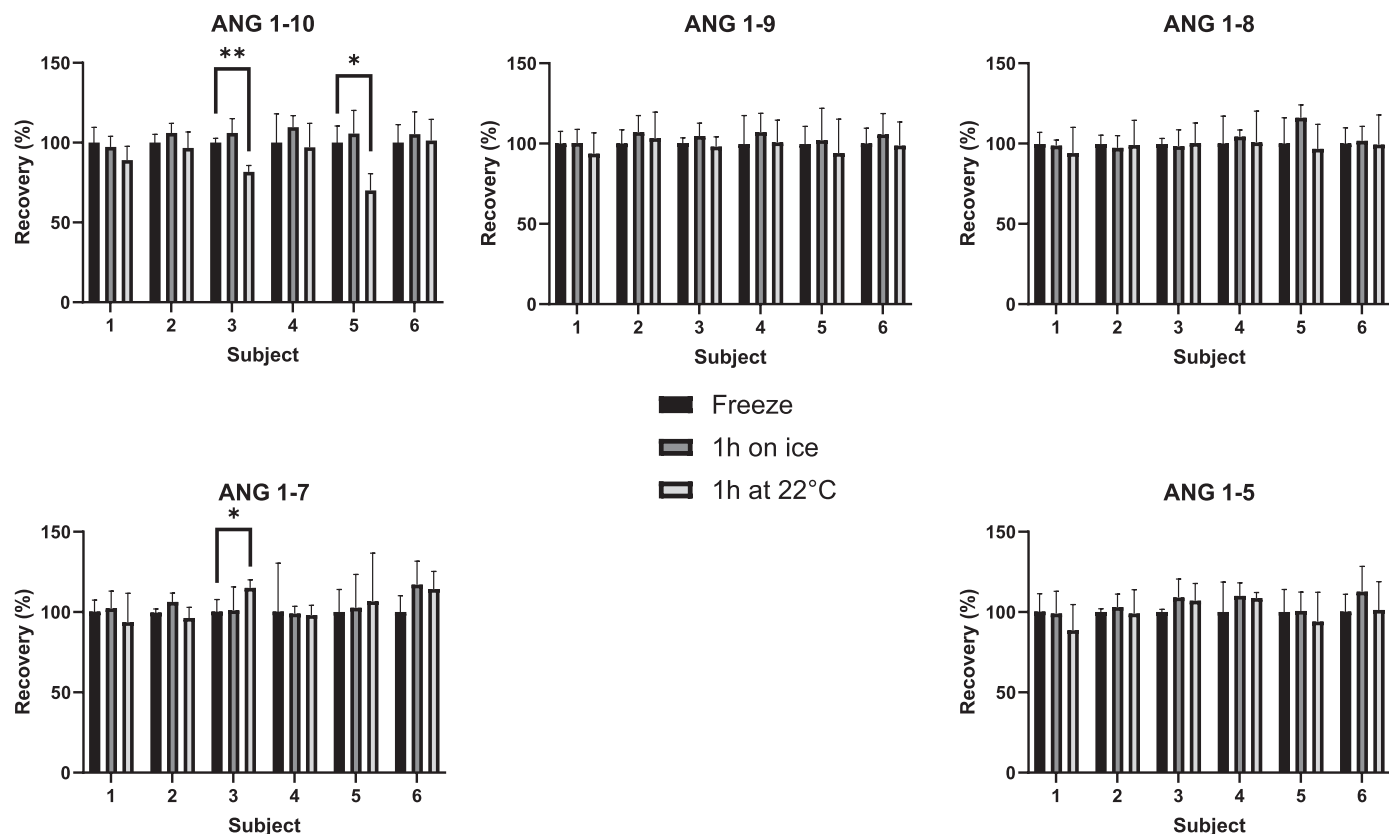


Fig. 3. : Recovery of ANGs from the plasma of six healthy donors (spiked with 1000 pM of each ANGs) in three conditions: stored at -80°C , incubated on ice for 1 hour, and incubated at 22°C for 1 hour. The inhibitor mix efficiently prevented the production or degradation of ANGs when the samples were incubated on ice, but showed limitations when the samples were incubated at 22°C . * $p \leq 0.05$; ** $p \leq 0.01$.

quantified 4 times over 1 month.

3. Results and discussion

3.1. Pre-analytical considerations

We developed the assay by following a dedicated tutorial for peptide analysis [31]. First, we evaluated adsorption of ANGs by performing a transfer test as proposed in this tutorial. Adsorption occurred in the 700 μL collection plate and in the Eppendorf tube used, resulting in a 20% loss after five transfers. We did not observe adsorption when we diluted ANGs in a solution of FA 0.1%–NG 0.1%. In addition, the solubilisation step of the dried extract after SPE induced a significant 50% loss when only water or FA 0.1% was used, but no loss when using MeOH 10%–FA 0.1%–NG 0.1%. We have already published the study of ANG adsorption elsewhere [31]. Second, similarly to other authors, we observed a strong influence of the anticoagulant on the results: heparinised tubes prevented proper detection of ANGs [20]. Finally, we performed a comprehensive study of ANG stability in EDTA plasma spiked with 1000 pM of ANGs incubated at 37°C for 0.5 and 2 hours (Fig. 2). We found that initially, renin activity induced a very important and fast production of ANG1–10, up to 20 times the initial concentration after incubation for 2 hours. In parallel, 50%–75% of ANG1–9 and ANG1–8 were degraded, and we observed significant production of ANG1–7. Of note, the ANG1–10 production did not induce a parallel increase in all other ANGs, suggesting that there is a different metabolic rate for renin, ACE, ACE2, and neprilysin. Therefore, the plasma concentrations would be completely linked to the inhibition of these proteases and to the time spent at a temperature allowing their activity.

During the method development, we tested many protease inhibitors to fully prevent any change in ANG plasma concentrations, by targeting

not only ACE, ACE2, neprilysin, and renin, but also other nonspecific proteases, as recommended by Maurer et al. [31]. Protease inhibitors must be chosen with particular care because they might induce an important matrix effect, resulting in interferences or reduced sensitivity. The optimal mix should contain as few protease inhibitors as possible while fully stabilising the peptides during the entire extraction procedure. The addition of the renin inhibitor aliskiren was required to prevent the generation of ANG1–10 from angiotensinogen. We tested different ACE inhibitors (enalapril, captopril, and quinapril) and selected captopril because it did not induce a significant matrix effect. To inhibit neprilysin, we tested several metalloprotease inhibitors – including EDTA, ethylene glycol-bis(β -aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA), 1,10-phenanthroline, and 2,2'-bipyridyl – and finally select both EDTA and 2,2'-bipyridyl. This mix of four protease inhibitors efficiently stabilised ANGs, but not equally in every plasma sample tested, highlighting the problem of protease activity inter-variability and the importance of comprehensive stability studies in various conditions and plasmas. Fig. 3 shows a significant 25% degradation of ANG1–10 after 1 hour at room temperature in two out of six plasma samples previously supplemented with the protease inhibitor mix ($p = 0.003$ and 0.025 , respectively), and a significant 15% production of ANG1–7 in one plasma sample ($p = 0.048$).

With this limitation in mind, we considered the stability to be sufficient when the samples are collected in pre-chilled EDTA tubes, stored on ice immediately after blood collection, and centrifuged within 30 minutes.

3.2. Assay development

Because we did not have blank plasma samples without detectable amounts of ANGs, we tested surrogate matrices to prepare the

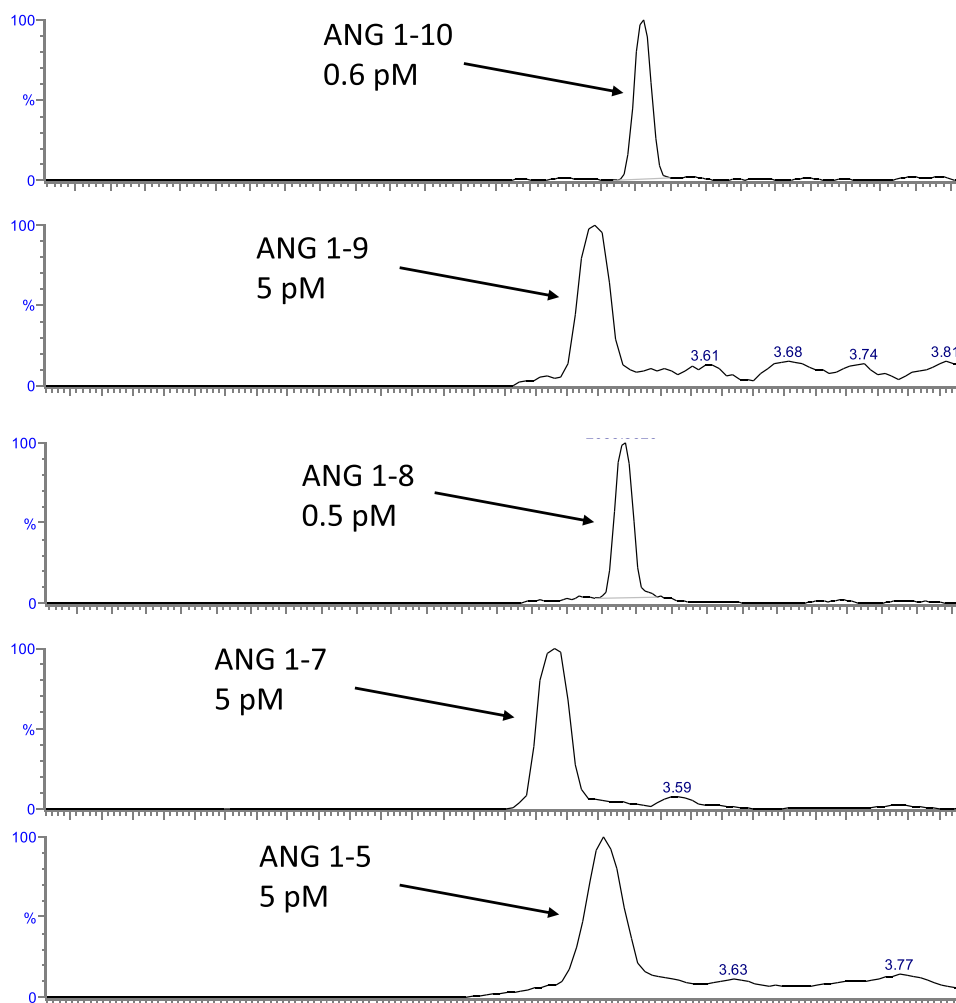


Fig. 4. : Representative micro-UHPLC-MS/MS chromatograms of the five ANGs at their respective LLOQ. ANG1-10 and ANG1-8 were recovered from a surrogate matrix composed of pooled plasma diluted 10 times. ANG1-9, ANG1-7, and ANG1-5 were recovered from plasma spiked with 5 pM of each ANG.

calibration curve. We compared the recoveries of the five ANGs spiked at 100 pM in the surrogate matrices and in plasma. We tested water, PBS with NG 0.1%, PBS with bovine serum albumin 0.5%, FA 0.1%–NG 0.1%, charcoal-stripped plasma, and pooled plasma diluted 100- and 10-fold with water. Among all, the latter showed the most similar properties to plasma, with a recovery increased by 1.5–2 times, while having low endogenous ANG concentrations. This matrix showed detectable amounts of ANG1-10 and ANG1-8 (0.84 and 0.81 pM for the one used for the method development; [Table S1](#)). We considered the remaining concentrations in the calibration curve by adding the endogenous concentration to the spiked ANG. Therefore, the exact ANG concentrations in the calibration curve changes each time a new batch of plasma is used to prepare the curve. We assessed linearity for all five peptides over the eight points of the calibration curve. The mean slope, intercept, and r^2 measured in three separate runs are shown in [Fig. 1S](#).

3.3. Analytical validation

We determined the LLOQ at 0.6, 5, 0.5, 5, and 5 pM for ANG1-10, ANG1-9, ANG1-8, ANG1-7, and ANG1-5, respectively. We used the same surrogate matrix used for the calibration curves to evaluate the LLOQ of ANG1-10 and ANG1-8 because blank plasma was not available. This is a limitation of our study that might result in an overestimation of the LLOQ for these two ANGs. [Fig. 4](#) shows chromatograms of the five ANGs at their respective LLOQ. ANG1-10 and ANG1-8 are in the surrogate matrix, and ANG1-9, ANG1-7, and ANG1-5 are in a plasma sample.

We evaluated the limit of detection (LOD) in the same matrices by estimating the lowest possible concentration that would produce a signal high enough to be separated from the background noise. The estimated LOD was < 0.1 pM for ANG1-10 and ANG1-8, < 0.5 pM for ANG1-9 and ANG1-7, and < 1 pM for ANG1-5. The intra- and inter-assay precision ranged from 4% to 22% and 14–28%, respectively ([Table S4](#)).

Recovery – calculated as the area ratio of the plasma of 3 healthy donors spiked with ANG at 100 pM – ranged from 5% to 20% ([Table S5](#)). The low recovery observed is easily explained by the aggressive sample preparation needed to obtain clean samples for the micro-UHPLC and the use of protease inhibitors that impacted the extraction. However, we did not further optimise the recovery because we could easily detect ANG1-10 and ANG1-8, while the other ANGs showed circulating concentrations way below the LLOQ. Thus, even 100% recovery would not have allowed us to detect ANG1-9, ANG1-7, and ANG1-5 at circulating concentrations. We evaluated the matrix effect on the same 3 plasma samples; it was highly dependent on the plasma state. For example, the matrix effect induced by haemolysis reduced the signal by a factor of 1.5–2, resulting in a higher LLOQ (data not shown). Thus, samples showing strong haemolysis and with concentrations close to the LLOQ should be analysed carefully.

Carryover was $< 10\%$ for each ANG, which is sufficiently low so that a blank does not need to be injected between each sample. We are confident that sample within the calibration range will not affect subsequent analysis.

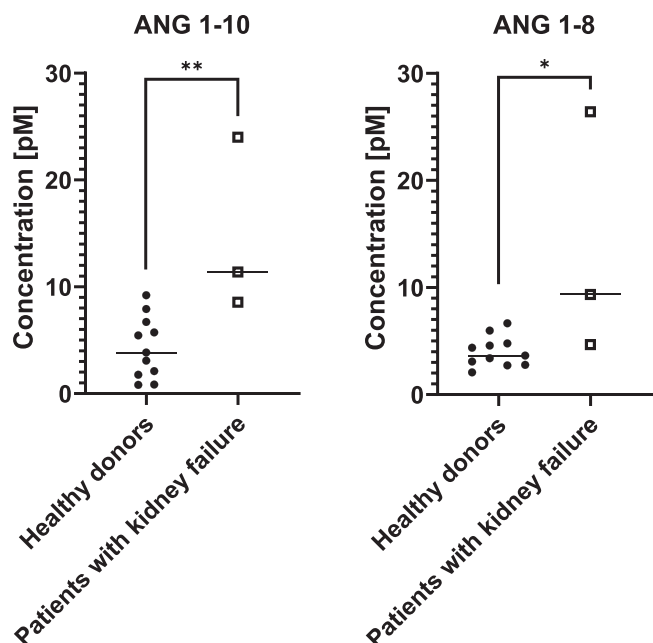


Fig. 5. : Plasma concentrations of ANG 1-10 and ANG 1-8 measured in 11 healthy donors and in 3 patients with kidney failure. * $p \leq 0.05$; ** $p \leq 0.01$.

Sample dilution with water allowed us to measure samples with concentrations higher than the highest point of the calibration curve or samples with a volume < 450 μL . The precision and accuracy were not changed provided that the final concentrations of the samples were within the calibration curve range (data not shown).

3.4. Stability

The freeze (-80°C for > 24 hours) and thaw (22°C for > 10 minutes) stability in plasma supplemented with protease inhibitors and after three cycles was between 84% and 116% for all the ANG1s spiked at the QC high concentrations. We assessed the short-term stability in plasma supplemented with protease inhibitors for 1 hour on ice (see Fig. 3). We assessed stability in the blood collection tube prior to the centrifugation for 30 minutes on ice (see Fig. 2S).

We also assessed the stability of ANG concentrations in plasma with and without protease inhibitors incubated at 4°C for 24 hours. The ANG concentrations were stable in the plasma samples that contained protease inhibitors. However, when there were no protease inhibitors, there was still significant ANG metabolism (see Fig. 3S).

Post-extracted samples showed no degradation over 72 hours compared with samples stored at -80°C (data not shown). We assessed long-term stability using QC samples measured over 1 month. The concentrations were stable, showing a CV < 20% (data not shown).

With the stability of ANG1s confirmed in plasma samples and after the extraction, we considered the pre-analytical stability to be suitable to perform the analysis in the usual conditions, provided that samples were supplemented with the dedicated protease inhibitor mix, cooled right before sampling, centrifuged within 30 minutes, and handled on ice during the extraction.

3.5. Measurement of ANG1s in samples from healthy volunteers

We used our successfully validated method to measure ANG1s in samples obtained from 11 healthy volunteers and 3 patients with kidney failure (Tables S6 and S7). We measured ANG1-10 and ANG1-8 in all samples (Fig. 5). In healthy donors, the median concentrations were 3.85 pM (IQR: 1.93–6.22) and 3.64 pM (IQR: 2.93–4.67), respectively. In patients with kidney failure, the median concentrations were 11.38

Correlation between ANG 1-10 and ANG 1-8

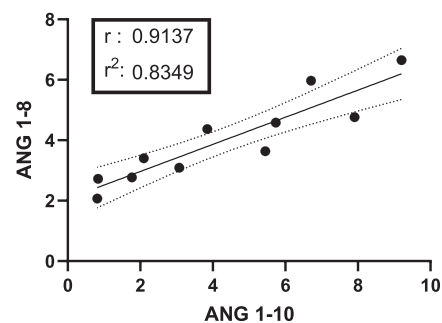


Fig. 6. : Correlation between the measured concentrations of ANG1-10 and ANG1-8 in 11 healthy donors. The dotted lines represent the error bars.

pM (IQR: 9.96–17.69) and 9.35 pM (IQR: 7.01–17.88), respectively. The mean concentration differences of ANG1-10 and ANG1-8 in the two populations were statistically significant ($p = 0.011$ and $p = 0.031$, respectively). Our data support a detectable modification of the ANG1s concentration in patients with kidney failure. However, the small cohort did not allow to highlight treatment (such as ACEi) or the impact of a recent dialysis that would explain these observations. Conversely, ANG1-9, ANG1-7, and ANG1-5 were not detectable in any of the samples, despite a LOD in the sub-picomolar range and despite controlled pre-analytical steps, ensuring the stability of all ANG1s from the sampling to the analysis.

There was a very strong positive correlation between the two peptides (Fig. 6, $r = 0.91$, $p < 0.0001$). We expected this outcome because ANG1-8 is mainly produced by the metabolism of ANG1-10 by ACE, directly linking the concentration of the two peptides.

For many years, ANG1s have been quantified in the plasma of healthy volunteers, and reference intervals have been determined. Table 1 reports the concentrations from 15 recent papers that aimed to quantify the circulating or equilibrium concentration of ANG1s. The latter corresponds to the concentrations measured after incubation of the plasma for 30 minutes at 37°C to allow the metabolism of the ANG1s by the RAAS to reach an equilibrium. According to these studies, ANG1s circulate at concentrations between 4 and 425 pM for ANG1-10, between < 3 and 51 pM for ANG1-9, between 1 and 142 pM for ANG1-8, between 1 and 122 pM for ANG1-7, and between < 2 and 90 pM for ANG1-5.

Table 1 shows a high variability in the reported range of circulating concentrations, with 2–3 orders of magnitude for each ANG peptide and highly variable concentrations within and between studies. In addition, none of these 15 studies described a comprehensive stability evaluation, and only one mentioned an adsorption test, while these parameters are known to have tremendous effects on recovered concentrations. The lack of such studies and the general lack of transparency have already been a subject of debate among authors regarding the quantification of ANG1s and other peptides [46–51]. In addition, the concentrations obtained using the so-called equilibrium concentration are in the range of the supposedly circulating concentrations. This finding supports post-sampling degradation of ANG1s in both techniques, leading to artificially high ANG1 concentrations. Thus, the strong limitations of these studies raise concerns about the reliability of the results. The concentration ranges are likely the consequence of inappropriate sample handling, poor stability control, and lack of specificity rather than population differences. These factors easily explain the high discrepancies in the concentrations reported within and between the 15 studies. Moreover, 5 of the 15 studies used methods other than MS; these other methods show poor selectivity between the ANG1 fragments [52].

Notable findings emerged from the stability study conducted with plasma without protease inhibitors (see Fig. 2). We observed a substantial increase in the production of ANG1-10, accompanied by a decrease in both ANG1-8 and ANG1-9, along with the generation of

Table 1

Review of 15 articles that aimed to quantify ANG1 in human plasma. The values in green are in range of the concentrations we found in the present study. For the comparison, when multiple populations were studied, only the reference population is included. When raw data were given, the median, Q1 and Q3 were calculated, and values below the LLOQ were set at half the provided LLOQ [33]. Furthermore, when different techniques were compared, only the results from the MS-based assay are reported. Finally, pg/mL were converted into pM [34–45].

	Concentration (pM)	Patient type	Stability study in sample matrix	Inactivation of plasma proteases	Analytical technique
<i>ANG1-10</i>					
Suzuki et al. [19]	8.1 ± 2.6	Healthy donors	Adsorption No stability study from collection to storage	EDTA, 1,10-phenanthroline, enalaprilat dihydrate, pepstatin A	MS
Kutz et al. [34]	66.8 ± 6.8 (Eq. Conc.)	COVID-19-negative patients	Not described	Not described	MS
Valle Martins et al. [35]	45.2 ± 10.8	COVID-19-negative patients without ACEi	Not described	Guanidine thiocyanate in 0.5% TFA	MS
Urwylter et al. [36]	245.6 ± 97.7 (Eq. Conc.)	Males with body mass index ≥ 30 kg/m ²	Not described	Not described	MS
Basu et al. [37]	6.0 (4.1–10.7)	Healthy donors	Not described	Attoquant Diagnostics mix*	MS
Matsui et al. [38]	230.6 ± 21.1	Healthy donors	Not described	Not described	MS
Chen et al. [39]	133.7 ± 22.9	Patients with hypertension	Data not shown	PMSF, SBTI and EDTA	MS
Shen et al. [40]	385.8 ± 30.9	Healthy donors	Not tested on samples	Fast treatment	MS
Campbell and Kladis [41]	17.4 ± 4.6	Healthy donors	Poorly described	Pepstatin, PMSF, and leupeptin in ethanol	RIA
Reyes-Engel et al. [42]	92.5 ± 102.1	Healthy donors	Not described	EDTA, <i>o</i> -phenanthroline, <i>p</i> -chloromercuribenzoic acid, and pepstatin A	RIA
Demeuse et al. [18]	318.3 (95.2–425.0)	Healthy donors	Only assessed at 4 °C in plasma	Fast treatment	MS
<i>ANG1-9</i>					
Amezcu-Guerra et al. [43]	34.9 (10.0–51.4)	COVID-19-positive patients who required hospitalisation	Not described	Not described	ELISA kit
Basu et al. [37]	< 3	Healthy donors	Not described	Attoquant Diagnostics mix*	MS
Shen et al. [40]	16.9 ± 4.2	Healthy donors	Not tested on samples	Fast treatment	MS
Demeuse et al. [18]	< 5	Healthy donors	Only assessed at 4 °C in plasma	Fast treatment	MS
<i>ANG1-8</i>					
Amezcu-Guerra et al. [43]	2.36 (0.99–4.76)	COVID-19-positive patients who required hospitalisation	Not described	Not described	CZE
Cohall et al. [44]	111.1 ± 141.8	Normotensive Afro-Caribbean Barbadians	Not described	1, 20 <i>o</i> -phenanthroline monohydrate, pepstatin, and sodium <i>p</i> -hydroxymercuribenzoate	RIA

(continued on next page)

Table 1 (continued)

Suzuki et al. [19]	2.6 ± 0.3	Healthy donors	Adsorption No stability study from collection to storage	EDTA, 1,10-phenanthroline, enalaprilat dihydrate, and pepstatin A	MS
Kutz et al. [34]	92.5 ± 6.4	COVID-19-negative patients	Not described	Not described	MS
Valle Martins et al. [35]	11.2 ± 2.07	COVID-19-negative patients without ACEi	Not described	Guanidine thiocyanate in 0.5% TFA	MS
Urwyler et al. [36]	212.6 ± 64.8 (Eq. Conc.)	Male with body mass index ≥ 30 kg/m ²	Not described	Not described	MS
Basu et al. [37]	5.1 (3.8–8.2)	Healthy donors	Not described	Attoquant Diagnostics mix*	MS
Matsui et al. [38]	39.2 ± 7.0	Healthy donors	Not described	Not described	MS
Khajeh Pour et al. [20]	0.87 ± 0.16	Patients with rheumatoid arthritis in remission stage	Not described	p-Hydroxymercuribenzoate, 1,10-phenanthroline, PMSF, pepstatin A, and EDTA	MS
Shen et al. [40]	86.0 ± 19.1	Healthy donors	Not tested on samples	Fast treatment	MS
Wai-Shan Lo et al. [21]	4.4–17.7 and 3.9–12.8	Healthy donors 21–30 and 31–60 years old	Poorly described	Not described	MS
Campbell and Kladis [41]	10.7 ± 3.4	Healthy donors	Poorly described	Pepstatin, PMSF, and leupeptin in ethanol	RIA
Reyes-Engel et al. [42]	22.4 ± 10	Healthy donors	Not described	EDTA, o-phenanthroline, p-chloromercuribenzoic acid, and pepstatin A	RIA
Ferrario et al. [45]	42.4 ± 1.7	Patients with hypertension	Not described	1, 20 o-phenanthroline monohydrate, pepstatin, and sodium p-hydroxymercuribenzoate	RIA
Demeuse et al. [18]	2.5 (2.5–5.1)	Healthy donors	Only assessed at 4°C in plasma	Fast treatment	MS
ANGI-7					
Amezcu-Guerra et al. [43]	0.56 (0.30–0.74)	COVID-19-positive patients who required hospitalisation	Not described	Not described	CZE
Cohall et al. [44]	76.5 ± 61.6	Normotensive Afro-Caribbean Barbadians	Not described	1, 20 o-phenanthroline monohydrate, pepstatin, and sodium p-hydroxymercuribenzoate	RIA
Suzuki et al. [19]	1.75 ± 0.7	Healthy donors	Adsorption No stability study from collection to storage	EDTA, 1,10-phenanthroline, enalaprilat dihydrate, and pepstatin A	MS
Kutz et al. [34]	7.6 ± 4.7 (Eq. Conc.)	COVID-19-negative patients	Not described	Not described	MS
Valle Martins et al. [35]	6.47 ± 1.03	COVID-19-negative patients without ACEi	Not described	Guanidine thiocyanate in 0.5% TFA	MS
Urwyler et al. [36]	17.2 ± 6.9 (Eq. Conc.)	Males with body mass index ≥ 30 kg/m ²	Not described	Not described	MS
Basu et al. [37]	< 2	Healthy donors	Not described	Attoquant Diagnostics mix*	MS
Khajeh Pour et al. [20]	7.6 ± 2.6	Patients with rheumatoid arthritis in remission stage	Not described	p-Hydroxymercuribenzoate, 1,10-phenanthroline, PMSF, pepstatin A, and EDTA	MS

Table 1 (continued)

Shen et al. [40]	77.9 ± 44.5	Healthy donors	Not tested on samples	Fast treatment	MS
Reyes-Engel et al. [42]	31.7 ± 27.5	Healthy donors	Not described	EDTA, <i>o</i> -phenanthroline, <i>p</i> -chloromercuribenzoic acid, and pepstatin A	RIA
Ferrario et al. [45]	13.1 ± 2.1	Patients with hypertension	Not described	1, 20 <i>o</i> -phenanthroline monohydrate, pepstatin, and Na <i>p</i> -hydroxymercuribenzoate	RIA
Demeuse et al. [18]	7.3 (3.3-9.9)	Healthy donors	Only assessed at 4°C in plasma	Fast treatment	MS
<i>ANG1-5</i>					
Kutz et al. [34]	6.6 ± 3.4 (Eq. Conc.)	COVID-19 negative patients	Not described	Not described	MS
Valle Martins et al. [35]	17.5 ± 6.29	COVID-19 negative patients without ACEi	Not described	Guanidine thiocyanate in 0.5% TFA	MS
Urwylker et al. [36]	7.3 ± 2. (Eq. Conc.)	Male with body mass index ≥ 30 kg/m ²	Not described	Not described	MS
Basu et al. [37]	< 2	Healthy donors	Not described	Attoquant Diagnostics mix*	MS
Shen et al. [40]	60.2 ± 30.1	Healthy donors	Not tested on samples	Fast treatment	MS

CZE, capillary zone electrophoresis; Eq. Conc., equilibrium concentration; PMSF, phenylmethylsulphonyl fluoride; RIA, radioimmunoassay; SBTI, soybean trypsin inhibitor, *, broad spectrum protease inhibitors against metalloproteases (EDTA, 1,10-phenanthroline), aspartic proteases (pepstatin A), cysteine proteases (*p*-hydroxymercuribenzoic acid), serine proteases (AEBSF), and specific inhibitors for renin and aminopeptidases A and N to a final concentration of 5% v/v.

ANG1–7. We wanted to see if this could have an impact not only on incubated samples but also on during samples collected and prepared in a conventional way. Thus, blood samples were collected from one healthy donor in three tubes containing no additional protease inhibitors and three tubes containing our mix of protease inhibitors. Tubes were centrifuged and aliquots were frozen at -80°C within 30 minutes. The samples were kept on ice for the whole extraction. ANG1–7 from each of the six tubes were then measured during the same run. The concentrations of ANG1–10 were 9 pM and 50 pM with and without inhibitors, respectively. The difference was statistically significant ($p = 0.0004$). The concentration of ANG1–8 was not statistically different ($p = 0.499$). Finally, ANG1–7 was detected at endogenous level in the plasma without inhibitors. The chromatograms obtained when the blood was sampled with and without the protease inhibitors are presented in Fig. 4S. The concentration of ANG1–7 evaluated at 2.6 pM was under the LLOQ so a high uncertainty accompanies this result.

Consequently, investigations conducted without protease inhibitors may encounter post-sampling concentration modifications. While the use of protease inhibitors can mitigate the metabolism and production of one or more ANG fragments, understanding which fragments are affected and the subsequent impact on concentrations remains elusive unless a comprehensive stability study is undertaken. For instance, our data suggest that with no protease inhibitors and provided that samples are centrifuged within 30 minutes, kept at -80°C and extracted on ice, concentrations of ANG1–10 and ANG1–7 are expected to increase 5 times and from < 0.5 pM to < 5 pM, respectively.

4. Conclusion

We have developed and validated an MS-based assay to quantify the circulating concentrations of ANG1–10, ANG1–9, ANG1–8, ANG1–7, and ANG1–5 in the plasma of healthy donors. For the first time, the development of the assay included a comprehensive optimisation of the pre-analytical conditions. To ensure the stability of the peptides, we used protease inhibitors to limit enzymatic degradation, and surfactants to reduce non-specific adsorption. The proven stability of ANG1–10 and ANG1–8 were measurable in the blood of 11 healthy donors at 3.85 and 3.64 pM, respectively. Moreover, there was a very strong positive correlation between the two ANG1–10 and ANG1–8, which we expected given that ANG1–10 is mainly metabolised to ANG1–8 by ACE. Conversely, ANG1–9, ANG1–7, and ANG1–5 were not detectable despite a sub-picomolar LOD.

We also provided a literature review regarding the reported circulating concentrations of ANG1–10, ANG1–9, ANG1–8, ANG1–7, and ANG1–5. Our findings stand in stark contrast to the concentrations reported in the previous studies: between 4 and 425 pM for ANG1–10, between < 3 and 51 pM for ANG1–9, between 1 and 142 pM for ANG1–8, between 1 and 122 pM for ANG1–7, and between < 2 and 90 pM for ANG1–5. The important discrepancies we observed among the studies – namely very heterogeneous circulating concentrations of ANG1–10, ANG1–9, ANG1–8, ANG1–7, and ANG1–5 – can be attributed to the absence of proper pre-analytical control. We did not find any publication assessing in sufficient detail ANG1–10 stability in plasma from sampling to analysis, which raises important concerns about the concentrations reported. On the contrary, our findings support circulating concentrations that are far lower what has been reported in literature and pave the way to a better understand ANG1–10 production.

We are confident that the widespread adoption of MS-based peptide assays will continue to emphasise the importance of solid pre-analytical considerations. With this in mind, we urge analysts to consider the latest published guidelines [28–30] and dedicated peptide protocols [22,31,53] to improve the quality of future research.

CRedit authorship contribution statement

Anke de Groot: Acquisition of data, Analysis and interpretation of data, Drafting of the manuscript, Critical revision of the manuscript for important intellectual content. **Jonathan Maurer:** Conception and design, Acquisition of data, Analysis and interpretation of data, Drafting of the manuscript, Critical revision of the manuscript for important intellectual content, Obtaining funding, Supervision. **Eric Grouzmann:** Conception and design, Critical revision of the manuscript for important intellectual content, Supervision. **Léon Martin:** Acquisition of data, Analysis and interpretation of data, Drafting of the manuscript, Critical revision of the manuscript for important intellectual content. **Philippe J. Eugster:** Conception and design, Critical revision of the manuscript for important intellectual content, Supervision. **Grégoire Wuerzner:** Critical revision of the manuscript for important intellectual content, Administrative, technical, or material support, Supervision.

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.

Acknowledgements

We acknowledge the Bryn Turner-Samuels Foundation (Lausanne, Switzerland) for funding Jonathan Maurer's doctoral thesis and Wendy Brito for the blood sampling of patients and healthy donors.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at [doi:10.1016/j.jpba.2024.116101](https://doi.org/10.1016/j.jpba.2024.116101).

References

- [1] S. Patel, et al., Renin-angiotensin-aldosterone (RAAS): The ubiquitous system for homeostasis and pathologies, *Biomed. Pharm.* 94 (2017) 317–325.
- [2] K.M. Mirabito Colafella, D.M. Bovee, A.H.J. Danser, The renin-angiotensin-aldosterone system and its therapeutic targets, *Exp. Eye Res* 186 (2019) 107680.
- [3] C.A. McKinney, et al., Angiotensin-(1-7) and angiotensin-(1-9): function in cardiac and vascular remodelling, *Clin. Sci. (Lond.)* 126 (12) (2014) 815–827.
- [4] A. Povlsen, et al., The vasoactive mas receptor in essential hypertension, *J. Clin. Med.* 9 (1) (2020) 267.
- [5] L. Yu, et al., Angiotensin-(1-5), an active mediator of renin-angiotensin system, stimulates ANP secretion via Mas receptor, *Peptides* 86 (2016) 33–41.
- [6] P. Balakumar, G. Jagadeesh, A century old renin-angiotensin system still grows with endless possibilities: AT1 receptor signaling cascades in cardiovascular pathophysiology, *Cell Signal* 26 (10) (2014) 2147–2160.
- [7] M.M. Ibrahim, RAS inhibition in hypertension, *J. Hum. Hypertens.* 20 (2) (2006) 101–108.
- [8] B.C. Rossier, M. Bochud, O. Devuyst, The hypertension pandemic: an evolutionary perspective, *Physiol. (Bethesda)* 32 (2) (2017) 112–125.
- [9] L. Te Riet, et al., Hypertension: renin-angiotensin-aldosterone system alterations, *Circ. Res* 116 (6) (2015) 960–975.
- [10] F. Zhang, et al., Effects of RAAS inhibitors in patients with kidney disease, *Curr. Hypertens. Rep.* 19 (9) (2017) 72.
- [11] C.M. Ferrario, Cardiac remodelling and RAS inhibition, *Ther. Adv. Cardiovasc. Dis.* 10 (3) (2016) 162–171.
- [12] M. Paz Ocaranza, et al., Counter-regulatory renin-angiotensin system in cardiovascular disease, *Nat. Rev. Cardiol.* 17 (2) (2020) 116–129.
- [13] J. Nüssberger, et al., Specific measurement of angiotensin metabolites and in vitro generated Angiotensin II in plasma, *Hypertension* (1986).
- [14] G. Antonelli, et al., Mass spectrometry or immunoassay: est modus in rebus, *Clin. Chem. Lab Med* 55 (9) (2017) 1243–1245.
- [15] N.A. Lacher, K.E. Garrison, S.M. Lunte, Separation and detection of angiotensin peptides by Cu(II) complexation and capillary electrophoresis with UV and electrochemical detection, *Electrophoresis* 23 (11) (2002) 1577–1584.
- [16] J. Castro-Perez, C. Prakash, Recent advances in mass spectrometric and other analytical techniques for the identification of drug metabolites, *Identif. Quantif. Drugs, Metab., Drug Metab. Enzym. Transp.* (2020) 39–71.
- [17] L. Bernstone, J.E. Adaway, B.G. Keevil, An LC-MS/MS assay for analysis of equilibrium angiotensin II in human serum, *Ann. Clin. Biochem.* 58 (5) (2021) 422–433.

- [18] J. Demeuse, et al., 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, *J. Chromatogr. B* 1232 (2024).
- [19] S. Suzuki, et al., Robust analysis of angiotensin peptides in human plasma: column switching-parallel LC/ESI-SRM/MS without adsorption or enzymatic decomposition, *Anal. Biochem* 630 (2021) 114327.
- [20] S. Khajeh Pour, et al., Plasma angiotensin peptides as biomarkers of rheumatoid arthritis are correlated with anti-ACE2 auto-antibodies level and disease intensity, *Inflammopharmacology* (2022).
- [21] Wai-Shan Lo, C., et al., Quantitation of plasma angiotensin II in healthy Chinese subjects by a validated liquid chromatography tandem mass spectrometry method, *Biomedical Chromatography*, 2022.
- [22] K. Maes, et al., Strategies to reduce aspecific adsorption of peptides and proteins in liquid chromatography–mass spectrometry based bioanalyses: an overview, *J. Chromatogr. A* 1358 (2014) 1–13.
- [23] L. Cui, K. Nithipatikom, W.B. Campbell, Simultaneous analysis of angiotensin peptides by LC–MS and LC–MS/MS: metabolism by bovine adrenal endothelial cells, *Anal. Biochem.* 369 (1) (2007) 27–33.
- [24] S. Schiffer, et al., Cardiovascular regulation in TGR(mREN2)27 rats: 24h variation in plasma catecholamines, angiotensin peptides, and telemetric heart rate variability, *Chrono-. Int* 18 (3) (2001) 461–474.
- [25] N. Ohashi, et al., Circadian rhythm of blood pressure and the renin-angiotensin system in the kidney, *Hypertens. Res* 40 (5) (2017) 413–422.
- [26] S. Patlar, S. Ünsal, RAA system and exercise relationship, *Turk. J. Sport Exerc.* (2019) 261–269.
- [27] L.M. Hilliard, et al., The "his and hers" of the renin-angiotensin system, *Curr. Hypertens. Rep.* 15 (1) (2013) 71–79.
- [28] FDA, Guidance for Industry - Bioanalytical Method Validation, in: U.S.F.a.D. Administration. (Ed.) 2018.
- [29] EMA, ICH guideline M10 on bioanalytical method validation and study sample analysis. (Ed.) 2022.
- [30] CLSI, C64 Quantitative Measurement of Proteins and Peptides by Mass Spectrometry. (Ed.) 2021.
- [31] J. Maurer, E. Grouzmann, P.J. Eugster, Tutorial review for peptide assays: an ounce of pre-analytics is worth a pound of cure, *J. Chromatogr. B Anal. Technol. Biomed. Life Sci.* 1229 (2023) 123904.
- [32] B.K. Matuszewski, M.L. Constanzer, C.M. Chavez-Eng, Strategies for the assessment of matrix effect in quantitative bioanalytical methods based on HPLC-MS/MS, *Anal. Chem.* (2003).
- [33] Johnson, J.R., *Methods for Handling Concentration Values Below the Limit of Quantification in PK Studies*. PhUSE US Connect, 2018.
- [34] A. Kutz, et al., Renin-angiotensin-aldosterone system peptide profiles in patients with COVID-19, *Eur. J. Endocrinol.* 184 (4) (2021) 543–552.
- [35] A.L. Valle Martins, et al., Increased circulating levels of angiotensin-(1-7) in severely ill COVID-19 patients, *ERJ Open Res* 7 (3) (2021).
- [36] S.A. Urwyler, et al., IL (Interleukin)-1 receptor antagonist increases Ang (Angiotensin [1–7]) and decreases blood pressure in obese individuals, *Hypertension* 75 (6) (2020) 1455–1463.
- [37] R. Basu, et al., Roles of angiotensin peptides and recombinant human ACE2 in heart failure, *J. Am. Coll. Cardiol.* 69 (7) (2017) 805–819.
- [38] T. Matsui, et al., Determination of angiotensin metabolites in human plasma by fluorimetric high-performance liquid chromatography using a heart-cut column-switching technique, *J. Chromatogr. B* (1999).
- [39] F. Chen, et al., A liquid chromatography-tandem mass spectrometry (LC-MS/MS)-based assay for simultaneous quantification of aldosterone, renin activity, and angiotensin II in human plasma, *J. Chromatogr. B* 1179 (2021) 122740.
- [40] Y. Shen, et al., Simultaneous determination of nine trace concentration angiotensin peptides in human serum using ultra high performance liquid chromatography with tandem mass spectrometry with sephadex LH-20 gel solid-phase extraction, *J. Sep. Sci.* 42 (13) (2019) 2247–2254.
- [41] D.J. Campbell, A. Kladis, Simultaneous radioimmunoassay of six angiotensin peptides in arterial and venous plasma of man, *J. Hypertens.* (1990).
- [42] A. Reyes-Engel, et al., Influence of gender and genetic variability on plasma angiotensin peptides, *JRAAS* (2006).
- [43] L.M. Amezcua-Guerra, et al., The prognostic importance of the angiotensin II/angiotensin-(1-7) ratio in patients with SARS-CoV-2 infection, *Ther. Adv. Respir. Dis.* 16 (2022), 17534666221122544.
- [44] D.H. Cohall, et al., Renin-angiotensin-aldosterone system gender differences in an Afro-Caribbean population, *J. Renin Angiotensin Aldosterone Syst.* 16 (3) (2015) 539–546.
- [45] C.M. Ferrario, J.A. Jessup, R.D. Smith, Hemodynamic and hormonal patterns of untreated essential hypertension in men and women, *Ther. Adv. Cardiovasc Dis.* 7 (6) (2013) 293–305.
- [46] J. Maurer, E. Grouzmann, P.J. Eugster, The road to reliable peptide assays is paved with good guidelines, *Clin. Endocrinol.* (2022).
- [47] E. Grouzmann, P.J. Eugster, LC-MS/MS peptide assay validation: a plea for robust stability studies, *Clin. Chem.* 68 (5) (2022) 727–728.
- [48] M.C. Chappell, Reply to "Letter to the editor: angiotensin quantification by mass spectrometry", *Am. J. Physiol. Heart Circ. Physiol.* 310 (3) (2016) H454.
- [49] A.H.J. Danser, M. Poglitsch, Letter to the Editor regarding "A microanalytical capillary electrophoresis mass spectrometry assay for quantifying angiotensin peptides in the brain", *Anal. Bioanal. Chem.* 411 (30) (2019), 8163–8163.
- [50] M. Poglitsch, E.D. Sturrock, A.H. Danser, Letter to the editor: angiotensin quantification by mass spectrometry, *Am. J. Physiol. Heart Circ. Physiol.* 310 (3) (2016) H452–H453.
- [51] M.C. Chappell, Biochemical evaluation of the renin-angiotensin system: the good, bad, and absolute? *Am. J. Physiol. Heart Circ. Physiol.* 310 (2) (2016) H137–H152.
- [52] M.C. Chappell, et al., Concerns on the specificity of commercial ELISAs for the measurement of Angiotensin (1-7) and Angiotensin II in human plasma, *Hypertension* 77 (3) (2021) e29–e31.
- [53] J. Harald, et al., Analytical procedures for quantification of peptides in pharmaceutical research by liquid chromatography-mass spectrometry, *Anal. Bioanal. Chem.* 378 (4) (2004) 883–897.