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Development of a sensitive, accurate and robust liquid chromatography/mass spectrometric method for profiling of angiotensin peptides in plasma and its application for atherosclerotic mice☆

Mariola Olkowicz[a,b,*], Adrianna Radulska[a], Joanna Suraj[c,d], Agnieszka Kij[d], Maria Walczak[c,d], Stefan Chlopicki[d,e], Ryszard T. Smolenski[a]

[a] Department of Biochemistry, Medical University of Gdansk, Debinki 1, 80-211 Gdansk, Poland
[b] Department of Biotechnology and Food Microbiology, Poznan University of Life Sciences, Wojska Polskiego 48, 60-627 Poznan, Poland
[c] Department of Pharmacokinetics and Physical Pharmacy, Jagiellonian University Medical College, Medyczna 9, 30-688 Krakow, Poland
[d] Jagiellonian Centre for Experimental Therapeutics (JCET), Jagiellonian University, Bobrzynskiego 14, 30-348 Krakow, Poland
[e] Department of Experimental Pharmacology, Jagiellonian University Medical College, Grzegorzecka 16, 31-531 Krakow, Poland

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ABSTRACT

Quantification of angiotensin (Ang) peptides in biological matrices is a challenge due to their low picomolar (pM) concentration and poor analytical performance of current methods. This work aimed to select an optimal strategy for liquid chromatography/mass spectrometry (LC/MS) quantification of major angiotensins in plasma of wild type and atherosclerotic mice.

Optimal LC/MS set-up for Ang quantification was chosen, based on analytical performance, from: nanoflow/orbitrap, nanoflow/triple quadrupole and preconcentration nanoflow/triple quadrupole. The best LC/MS configuration (preconcentration nanoflow/triple quadrupole) was validated and used for measurement of angiotensins (Ang I, II, III, IV and (1–7)) in plasma of 6-month-old atherosclerotic apolipoprotein E/LDL receptor double knock-outs (ApoE/LDLR (−/−)) and wild type C57BL/6J (WT) mice.

The method established for Ang quantification was selective, accurate and highly sensitive with LLOQ of 5 pg mL^{-1}. The peak area intra-day precisions for Ang II and Ang-(1–7) were in the range 3.0–5.1 and 3.5–5.8, respectively, with corresponding accuracy of 95.4–103.5% and 95.6–106.3%. Plasma angiotensin profile was substantially modified in ApoE/LDLR knock-out mice with increase in concentration of Ang II from 37.6 ± 21.3 pg mL^{-1} in WT to 200.2 ± 47.6 pg mL^{-1}. Concentrations of Ang I, III and IV were also increased 3–10 fold in ApoE/LDLR (−/−) mice while that of Ang-(1–7) was unchanged.

We conclude that the method developed could be effectively used for accurate, comprehensive profiling of angiotensin peptides in mouse plasma. We identified substantial changes in renin–angiotensin system in a genetic mouse model of atherosclerosis consistent with the overactivation of angiotensin converting enzyme (ACE) and the impairment of ACE2.

1. Introduction

The renin–angiotensin system (RAS) and its effector molecules, angiotensin peptides, are known to exert a wide variety of functions in the cardiovascular and renal systems [1,2]. Activation of this system, especially due to increases in angiotensin II (Ang II) concentration has fundamental role in the pathological myocardial remodeling, progression of heart failure, diabetes and *atherothrombosis* [3,4]. Considerable research effort over the last decades was therefore focused on regulation of the RAS that revealed its complexity involving multiple alternate pathways and feedback mechanisms operating locally as well as systemically [5,6].

Ang II is considered as the primary effector molecule of the RAS that is best known for its important role in regulating blood pressure and renal sodium absorption [7]. Physiological effects of Ang II

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* Corresponding author at: Department of Biochemistry, Medical University of Gdansk, Debinki 1, 80-211 Gdansk, Poland. Tel.: +48 58 349 1464; fax: +48 58 349 1460.
E-mail address: m.olkowicz@gumed.edu.pl (M. Olkowicz).

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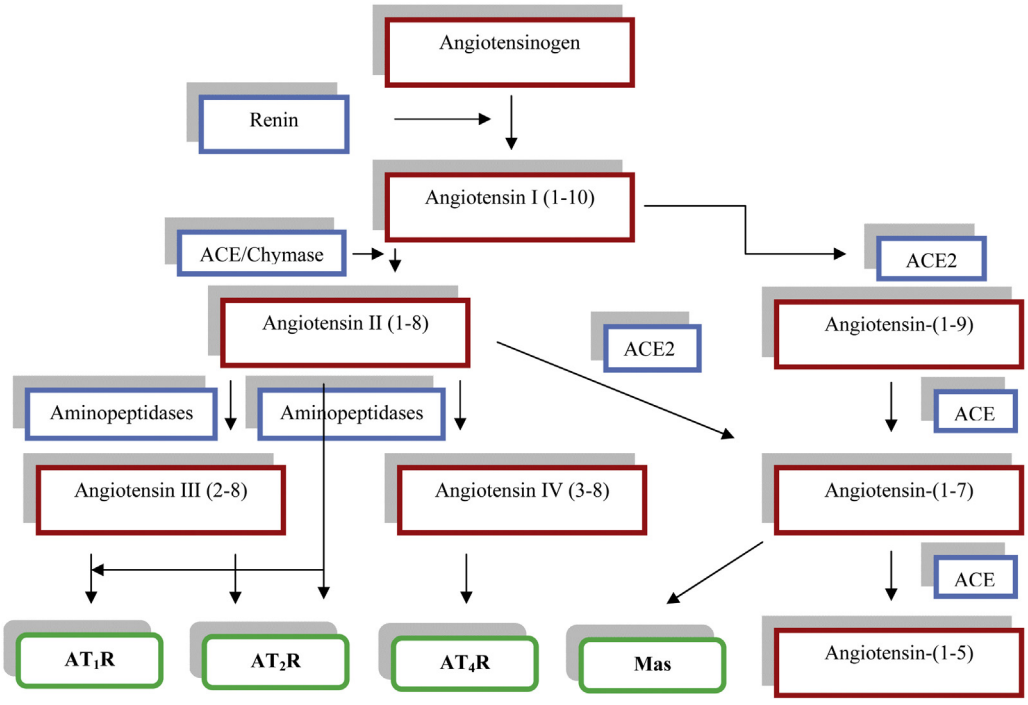

Fig. 1. Angiotensin I metabolism pathway. ACE – angiotensin converting enzyme; ACE2 – angiotensin converting enzyme 2; AT_1R – angiotensin II type 1 receptor; AT_2R – angiotensin II type 2 receptor; AT_4R – angiotensin type 4 receptor; Mas – G protein-coupled Ang-(1–7) receptor.

are exerted by binding to two types of G-protein coupled receptors: AT_1R and AT_2R found at different ratios in various tissues. Activation of the AT_1R, which is highly expressed in vascular wall and the heart, results in vasoconstriction, cell proliferation, hypertrophy and endothelial dysfunction. On the other hand, binding of Ang II to AT_2R counteracts the AT_1R-mediated deleterious effects, resulting in vasodilation and anti-hypertrophic effects linked to bradykinin and NO formation. As presented on Fig. 1 Ang II can be further metabolized to several active peptides, such as Ang-(1–7), Ang III (2–8) and Ang IV (3–8), that are also important components of the RA system [8,9]. Among the new RAS components the angiotensin converting enzyme 2 (ACE2)/Ang-(1–7)/G protein-coupled receptor Mas axis that exerts important cardiovascular effects deserves special attention [10]. This system was proved to be an important negative regulator of the ACE/Ang II/AT_1R axis in the development of cardiovascular diseases. Blockade of the AT_1R, resulting in an increase in Ang II concentration, was suggested to stimulate cardiac AT_2R, or accelerate conversion of Ang II to Ang-(1–7), which can provide beneficial therapeutic outcomes [11]. Therefore, studies on the formation of the angiotensin peptides or the regulation of ACE/ACE2 activity ratio are crucial for better understanding of mechanisms of cardiovascular diseases and identification of novel therapies targeting the RAS.

Physiological concentrations of angiotensin peptides in biological tissues/fluids are extremely low, normally in the picomolar range [12,13] and their accurate measurements require sensitive and specific methods. So far, analysis of angiotensin peptides has been usually performed using HPLC combined with radioimmunoassay (RIA) [14,15]. However, this technique has many drawbacks: predominantly is time-consuming and requires use of radiolabeled peptides. Therefore, there is a clear need for better analytical solution. Mass spectrometry hyphenated to high performance liquid chromatography could provide high selectivity and sensitivity for quantitative analysis of peptides. While procedures based on selected reaction monitoring, performed using triple quadrupole mass spectrometers are believed to offer best sensitivity [16], several alternatives recently emerged that apply high resolution mass spectrometry [17].

The aim of this study was to develop the LC/MS method for simultaneous detection and quantification of main products of angiotensin I metabolism in complex biological sample such as plasma. Three analytical solutions based on two different mass spectrometry (MS) techniques (high resolution MS and tandem MS) were critically compared. The most selective, sensitive and accurate procedure was tested for analytical quality and employed to quantify five Ang peptides (Ang I, II, III, IV and (1–7)) in plasma of wild type and atherosclerotic ApoE/LDLR (−/−) mice.

2. Material and methods

2.1. Chemicals

Peptide standards for: Ang I (1–10), Ang II (1–8), Ang III (2–8), Ang IV (3–8), Ang-(1–7) and [Asn^1, Val^5]-Ang II were purchased from Sigma–Aldrich (St. Louis, MO, USA), whereas ([ring-D_5]Phe^8)-Ang II was from Bachem (Bubendorf, Switzerland). PicoSure peptide standard was supplied by New Objective (Woburn, USA) and contained the equimolar mixture of synthetic peptides with the following sequences: (1) LGGGPGGGDGSR, (2) LGGGPGGGDFR, (3) LLGGPGGDFR, (4) EHWSYGLRPG, (5) DRVYIHPF, (6) LLLGPGDFR, (7) LLLPLDFR, (8) LLLLPLDFR. HPLC-grade acetonitrile (ACN), methanol (MeOH), acetic (glacial) and formic acid were obtained from Sigma–Aldrich (St. Louis, MO, USA). For the preparation of the samples and mobile phases ultrafiltered and deionized water from Barnstead Nanopure system was used. The filter membranes Vivaspin® 500 (polyethersulfone (PES) membrane, 5,000 molecular weight cut-off (MWCO)) were supplied by Sartorius (Göttingen, Germany), the Microcon® ultrafiltration devices (regenerated cellulose (RC) membrane, 10,000 MWCO) were purchased from Millipore (Bedford, MA, USA) and Roti®-Spin ultrafiltration concentrators (PES membrane, 10,000 MWCO) were from Carl Roth GmbH (Karlsruhe, Germany). The stock solution of each standard

at the concentration of 1 mg mL^{-1} was prepared in a glass vial, deionized water and kept frozen at −80 °C until used. The working standard solutions were prepared by dilution of the stock solutions with deionized water to obtain concentration in the range of 100 pg mL^{-1}–10,000 pg mL^{-1}.

2.2. Plasma collection and storage

The whole blood was collected by cardiac puncture from 6-month-old male ApoE/LDLR double knock-out (n = 8) and wild type C57BL/6J mice (n = 5) under isoflurane anesthesia. The study was approved by the Ethics Committee on Animal Experimentation at the Medical University of Gdansk. The blood samples were immediately mixed with protease inhibitor cocktail (Sigma–Aldrich) in a ratio of 5:1 and centrifuged at 1,000 × g for 15 min to isolate plasma. After the centrifugation, the resulting plasma was transferred into the Protein LoBind tubes (Eppendorf, Hamburg, Germany), split into aliquots and stored at −80 °C until further use. For LC/MS analysis, the aliquots were thawed at 4 °C, allowed to warm at room temperature and clarified by centrifugation, at 14,000 × g for 15 min, to sediment possibly insoluble particles.

2.3. Optimization of LC/MS configuration and analysis conditions

Three methods were established, based on different LC/MS set-ups and compared in terms of feasibility, specificity, sensitivity and linearity. In general, the instrument platforms consisted of a reversed phase nanoflow liquid chromatography (RPLC) system (typically 75 μm × 10 or 15 cm) coupled on-line to a mass spectrometer via a positive nanoelectrospray ionization (nanoESI) source. The three LC/MS platforms as presented in Table 1 were:

Set-up 1 (LC-FS-HRMS). System used was UltiMate 3000 Nano Quaternary LC (Dionex, Thermo Scientific, San Jose, CA, USA) interfaced via a PicoChip™ nanospray source (New Objective, Inc., Woburn, MA, USA) to a LTQ XL hybrid ion trap-Orbitrap Discovery mass spectrometer (Thermo Scientific). This LC-MS system included quaternary low-pressure gradient pump and an autosampler equipped with a 1 μL sample loop, coupled to a high-resolution/accurate mass spectrometer. To generate nanoflow rates appropriate UltiFlow split technology was used. PicoChip system, utilized for separation and as ion source (New Objective, Woburn, USA), consisted of a fused silica column packed with C18 resin. The MS scans recording was performed in the Fourier transform (FT) cell from a mass range of 300 to 1,500 m/z with the resolution set to 30,000 (FWHM) at 400 m/z and the automatic gain control (AGC) set to 1×10^6 ions. Extracted ion chromatograms (XIC) of target peptides were constructed with an optimized mass extraction window (MEW) 5 ppm. The LC/MS system, data acquisition and processing were managed by the Xcalibur software (version 2.1, Thermo Scientific).

Set-up 2 (Direct injection LC-MS/MS). The system included UltiMate 3000 Rapid Separation nanoLC system (Dionex, Thermo Scientific) interfaced via a PicoChip™ nanospray source (New Objective, Inc.) to a TSQ Vantage triple quadrupole mass spectrometer (Thermo Scientific). To determine the best selected reaction monitoring (MRM) transitions for each peptide, mass spectrometric conditions were optimized for synthetic peptides by infusing standard solution (10 μg mL^{-1}) prepared in a H_2O/ACN (50:50, v/v) mixture acidified with acetic acid (1%, v/v) at a flow rate of 15 μL min^{-1}. Peptides were ionized using the Heated Electrospray-II (HESI-II) probe and standard electrospray Ion-Max source. Emitter voltage (3,500 V), auxiliary/sheath gas pressure (20 units), vaporizer temperature (200 °C), and probe position were tuned manually, based on the optimal total ion current (TIC). Capillary temperature was set to 250 °C. The Full MS spectrum of each peptide was then acquired using the full scan and Q3MS scan mode (200–1,500 m/z). The most intense parent ions were selected for further fragmentation with the use of automated MRM Optimization mode. In general, mainly doubly charged $[M+2H]^{2+}$ ions were selected as precursor ions, whereas single charged b or y-ions with optimal MS response were selected as product ions for quantitation (quantifier) and confirmation (qualifier). All peptides were measured with Q1 and Q3 set to unit resolution (0.7 full width at half maximum, FWHM). The LC/MS system was controlled by the Xcalibur software (v. 2.1, Thermo Scientific). Table 2 summarizes in details the sequences of the peptides, precursor and product ions and collision energy settings.

Set-up 3 (Preconcentration LC-MS/MS). The same system that was used for direct injection mode was applied with the additional application of high-pressure 10-port switching valves, an autosampler equipped with a 20 μL sample loop and a ChipMate nanoelectrospray ion source (Advion, USA) in a place of the PicoChip. Analytical method details as well as the MS/MS transitions monitored are presented in Tables 1 and 2 respectively.

2.4. Optimization of sample pretreatment

Two different strategies for sample pretreatment were evaluated: centrifugal ultrafiltration (UF) and protein precipitation (PPT). The filter membranes were prepared according to the manufacturer's instructions. Various filtration devices were analyzed: Vivaspin® 500 (5 kDa, PES, Sartorius), Microcon® YM-10 (10 kDa, RC, Millipore) and Roti®-Spin 10 (10 kDa, PES, Carl Roth). In each case, 45 μL of plasma sample, fortified with 5 μL of the working standard solution of peptides (1,000 pg mL^{-1}), was diluted with 450 μL 5% (v/v) aqueous ACN and centrifuged at 2,000 × g for 30 min with the different filtration devices. Filtrates obtained were lyophilized to dryness and in the next step re-suspended in 25 μL of 1% aqueous acetic acid solution, before LC/MS analysis. Whereas, as the main protein-precipitating agent, acetonitrile (neutral or acidified) in various proportions to the samples (2:1, 4:1, v:v) was used. Sample clean-up procedure using this approach was as follows. 25 μL of mouse plasma was spiked with the peptides working standard solution (200 pg mL^{-1}) keeping the ratio: 1:1 (v:v). In the next step, blank ice-cold ACN (100 μL or 200 μL) was added and the stirred mixture was allowed to stand at −20 °C for 30 min. The precipitated proteins were removed by centrifugation at 15,000 × g for 15 min and the collected supernatant was evaporated to dryness. The dry residue obtained was reconstituted with 25 μL of H_2O acidified with 1% (v/v) acetic acid and analyzed using appropriate LC/MS system.

In the case of each of prefractionation methods, process recovery of angiotensin peptides from mouse plasma was calculated by comparing peak areas obtained from processed samples with those achieved after direct injections of standard solutions at the equivalent (200 pg mL^{-1}) concentration. Since the peptides of interest are present endogenously, samples of blank plasma were earlier analyzed to obtain the basic levels which were subtracted in all calculations used.

2.5. Validation of analytical method

2.5.1. System suitability test

System performance of the 3rd developed LC/MS method (as well as other described in this paper) was checked with a PicoSure test standard (New Objective, USA) that consisted of the equimolar mixture of 8 synthetic peptides (100 pmol of each/vial) dissolved in a solution of water/acetic acid (99:1, v:v). The gradient elution profile was: 2–75% B in 35 min, 75% B for 2 min, returning to 2% B in 2 min and 2% B was maintained until the end of the run (until

Table 1
LC/MS parameters of the three methods: high-resolution full scan LC/MS (LC-FS-HRMS), direct injection and preconcentration LC-MS/MS operating in multiple reaction monitoring (MRM) mode used for determination of angiotensin peptides.

	I SET-UP *LC-FS-HRMS*	II SET-UP *Direct injection* *LC-MRM-MS*	III SET-UP *Preconcentration* *LC-MRM-MS*
Column injection mode	Direct injection	Direct injection	Injection with the step of sample preconcentration; a trapping column – Acclaim PepMap100 C18, 2 cm × 75 μm, 3 μm, 100 Å (Thermo); mobile phase: ACN/H_2O 2/98 (v/v) containing 1% (v/v) acetic acid – delivered for 5 min at a flow rate of 5 μL min^{-1}
Analytical column	ProteoPep™ II, C18, 100 mm × 75 μm I.D., 5 μm, 300 Å (New Objective)	ProteoPep™ II, C18, 100 mm × 75 μm I.D., 5 μm, 300 Å (New Objective)	Acclaim PepMap100 RSLC C18, 150 mm × 75 μm I.D., 2 μm, 100 Å (Thermo)
Mobile phase	A – acetic acid (1%, v/v) in water; B – acetic acid (1%, v/v) in acetonitrile	A – acetic acid (1%, v/v) in water; B – acetic acid (1%, v/v) in acetonitrile	A – acetic acid (1%, v/v) in water; B – acetic acid (1%, v/v) in acetonitrile
Gradient elution profile/program	2–98% B, 0–25 min; 98% B, 25–35 min; 98–2% B, 35–40 min; 2% B, 40–55 min	2–98% B, 0–20 min; 98% B, 20–25 min; 98–2% B, 25–26 min; 2% B, 26–40 min	2% B, 0–5 min; 2–98% B, 5–25 min; 98% B, 25–30 min; 98–2% B, 30–32 min; 2% B, 32–50 min
Flow rate	400 nL min^{-1}	600 nL min^{-1}	300 nL min^{-1}
Column oven temperature	–	–	33 °C
Injection volume; injection mode	1 μL; full loop	1 μL; full loop	2 μL; partial loop
ESI capillary I.D./tip (μm)	75/15	75/15	50/15
Capillary voltage (kV)	2.1	2.1	2.1
Nebulizing gas flow (au.)	–	–	–
Ion tube temp. (°C)	200	200	200

50 min). The mass spectrometric conditions were: capillary voltage: 2.0 kV, temperature of ion transfer tube: 200 °C.

2.5.2. Linearity and sensitivity of the method

The linearity of the method was determined by analysis of five calibration curves, received for each analyte. Calibration standards (CS) and quality control (QC) samples were prepared by spiking 25 μL (pooled blank) plasma with 20 μL appropriate working standard solution and 5 μL IS – [Asn^1, Val^5]-Ang II (2,500 pg mL^{-1}). Calibration curve standards were made at: 5; 10; 50; 100; 250; 400; 500 pg mL^{-1} (IS – 250 pg mL^{-1}) concentrations, respectively. CS at seven different concentration levels were deproteinized with acetonitrile and assayed. The calibration curves were constructed by plotting the peak-area ratios of the analytes to the IS vs. the theoretical concentrations of the analytes, using weighted least squares linear regression (the weighting factor was $1/x$, where x was the concentration of the analyte). The LLOQ was defined as the lowest concentration of the analyte that yielded a signal-to-noise (S/N) ratio ≥10 with acceptable accuracy (within ±20%) and precision (≤20%).

2.5.3. Precision and accuracy, process recoveries

The accuracy and precision of the method were calculated from results of the analysis of QC samples at high (400 pg mL^{-1}), middle (250 pg mL^{-1}) and low (10 pg mL^{-1}) concentration levels prepared in plasma and extracted by means of protein precipitation on three different consecutive days. The accuracy as well as the intra- and inter-day precision were determined by analyzing five replicates of each QC sample level along with one standard curve on each of 3 days. Accuracy was evaluated using a following formula for calculating the recovery of endogenous substances in their biological matrices: [(mean found concentration − basal concentration)/theoretical concentration] × 100. Acceptable criteria for accuracy and precision were within ±15% relative error (*RE*) from the nominal values and within ±15% relative standard deviation (*RSD*) except for LLOQ, where its value should not deviate

Table 2
Molecular description and multiple reaction monitoring (MRM) settings for detection of angiotensin peptides.

Peptide	Amino acid sequence	MW (Da)	Precursor ion (*m*/*z*)	Charge state	Product ion[a] (*m*/*z*)	Collision energy (eV)
Ang I (1–10)	DRVYIHPFHL	1295.7	432.9	3+	**647.2**	17
					109.8	32
Ang II (1–8)	DRVYIHPF	1045.5	523.9	2+	**784.3**	19
					262.7	20
Ang-(1–7)	DRVYIHP	898.5	450.2	2+	**647.4**	19
					110.0	28
Ang III (2–8)	RVYIHPF	930.5	466.2	2+	**669.5**	17
					263.0	19
Ang IV (3–8)	VYIHPF	774.4	388.2	2+	**136.1**	21
					110.1	28
[Asn^1, Val^5]–Ang II (IS)	NRVYVHPF	1030.5	516.2	2+	**769.3**	19
					263.3	20

[a] – Two transitions for each peptide were monitored. LOQ values (presented in Table 4) as well as transition used for the quantification of each peptide in biological matrix refer to the transition in bold.

by more than 20%. These QC samples were assessed in each run along with biological samples to monitor the performance of the assay and to verify reliability of the analysis results obtained from the biological samples. Process recoveries were evaluated by direct comparison of the peak areas of the analytes extracted from plasma samples with those from the pure standard solution containing equivalent amounts of the analytes by analyzing five replicates of QC samples at low and high concentrations.

2.5.4. Stability of the analytes

The stability of the analytes in mouse plasma was assessed under a variety of storage and handling conditions at low and high concentrations. The short-term temperature stability was determined by analyzing low and high QC samples left at room temperature for 3 h. The long-term stability was determined by analyzing QC samples left at −80 °C for 1 month. The autosampler stability was assessed by analyzing low and high QC samples left in the autosampler at 4 °C for 12 h. Finally, freeze-thaw stability was verified through three cycles of freezing-thawing (−80 °C to room temperature as one cycle). Stability data were expressed as the percentage of the mean calculated vs. actual concentrations. The peptides were considered stable when the percentage deviation was within ±15%.

2.6. Data analysis

Peak finding, filtering, and alignment as well as scaling (to internal standard) were carried out using Xcalibur QuanBrowser software, version 2.1 (Thermo) capable of processing MRM data. Unless otherwise stated, default preprocessing parameters were as follows: smoothing half-width 10 points, baseline subtraction window 1.0 min, noise percentage 50%, peak-splitting factor 4, minimum required intensity 500, minimum peak width 3 points, minimum signal-to-noise 5.0, retention time tolerance 1.0 min. The levels of five angiotensin peptides were determined on the basis of the ratio of the peak areas of analyte and the internal standard. Quantification of the peptides was carried out by means of the standard addition calibration method. Concentrations of peptides in plasma were compared using Student's *t*-test to indicate any statistically significant difference between the values obtained for two studied groups. $P < 0.05$ was regarded as significant.

3. Results and discussion

This study advanced analytical potential for monitoring changes in renin–angiotensin system. Several innovations in nanospray LC-MS or LC-MS/MS methods were evaluated for simultaneous analysis of five angiotensin peptides including Ang I, Ang II, Ang III, Ang IV and Ang-(1–7). The most selective, sensitive and accurate set-up was validated and applied for quantification in mouse plasma. Analytical performance was superior to any previously published method. Comparison of angiotensin profile in atherosclerotic ApoE/LDLR (−/−) and wild type mice highlighted substantial increase in Ang I, Ang II, Ang III, Ang IV concentration without changes in Ang-(1–7). These results suggest a significant increase in ACE/ACE2 activity ratio in the mouse model of atherosclerosis compatible with known anti-atherosclerotic activity of ACE inhibitors [18].

3.1. LC/MS method development and optimization

A targeted LC-MS/MS approach rapidly emerged as a favorable quantitative solution for monitoring of biomarkers in biological fluids [16]. However, this approach requires MS/MS methods to be adopted for each analyte and with large number of analytes deficiencies of the approach such as long method development and decreased sensitivity for individual compounds become evident. As the number of analytes increases, the MS/MS method development becomes labor intensive, and the benefits of using a triple quadrupole diminish. In contrast, high resolution mass spectrometry (HR/AM) provides good-quality, quantitative data without the need to optimize MS/MS conditions for each analyte [17]. On the other hand, selectivity, provided by an accurate mass measurement and the use of narrow mass windows based on compounds exact mass, may not be sufficient for conducting specific analyses in complex biological matrices. Therefore, initial aim of this study was to compare different detection methods/system configurations and to identify the best solution for quantification of low-level analytes in complex biological samples.

The quantitative performance of the LTQ XL Orbitrap Discovery MS was compared with a triple quadrupole mass spectrometer (TQ-MS), TSQ Vantage, operating in the MRM mode. In all the method tested, chromatographic parameters (i.e., type of sorbent, type of mobile phase, elution profile) were similar to eliminate their effects on the results obtained. Fig. S1 (Supplementary Data) presents representative LC/MS and LC-MS/MS chromatograms of angiotensin peptides recorded in full scan (FS) and MRM mode. The performance of the HRFS-MS and MS/MS (in two configurations) was assessed regarding linearity, limits of quantification (LOQs), repeatability and selectivity with results presented in Table 3.

The linearity of the calibration curves was tested in the range relevant to the angiotensin peptide concentration in blood samples. Within ranges studied (50–10,000 pg mL^{-1} for HRFS, 25–1,000 pg mL^{-1} and 5–500 pg mL^{-1} for MS/MS modes), all target molecules exhibited good linearity with *r*-squared values >0.995.

The best sensitivity regarding LOQ values, was recorded for MS/MS method with sample preconcentration (5 pg mL^{-1}) confirming that tandem mass spectrometry (based on quadrupole technology) is more sensitive than Orbitrap system with number of analytes utilized in this study, and the use of preconcentration step only offers further gain in sensitivity. With increased number of analytes and more transitions to be monitored in the MS/MS mode, the dwell times would be shorter and sensitivity deteriorated.

Precision (repeatability) of the methods (tested at 200 pg mL^{-1} (set-up 1) and 100 pg mL^{-1} (set-up 2 and 3) concentration levels) was comparable between the three detection methods. Selectivity (an important parameter of method performance, especially when the difficult matrixes are analyzed) was slightly better for tandem MS technology. In the MRM approach, selectivity/specificity is achieved by removal of background ions in the first quadrupole and transmissions of the unique fragments in the second analyzer. Although this approach is very specific, its nature leads to the loss of valuable information about the composition of the sample as only specific ion transitions are monitored. In the full scan MS approach (using a single-stage Orbitrap mass spectrometer) specificity is obtained by stable high-resolution (up to 30,000 at 400 m/z) and accurate mass (<5 ppm with external calibration) performance. Despite the uniqueness of such a solution, HRFS still suffers from false positive results (data not shown). Such conclusions are in good agreement with previous data [17,19,20]. As described previously [17,20], HRFS MS is also not equally matrix-tolerant as quadrupole-based MS/MS. Some limitation may be insufficient specificity of the tested high-resolution instrument. Other authors have demonstrated that the latest generations of HR instruments with improved resolution (up to 100,000 at 400 m/z) and stability of accurate mass measurements can produce an equal or superior selectivity compared with unit mass resolution MS/MS [20]. In the present study, however, the best sensitivity and precision were achieved with the use of third LC/MS configuration and the method based on this set-up was used for further studies.

Table 3
Comparison between the high-resolution full scan LC/MS (LC-FS-HRMS), direct injection and preconcentration LC-MS/MS operating in multiple reaction monitoring (MRM) mode in terms of retention times (min, $\bar{x} \pm$ SD, $n = 5$), precision (%, $n = 5$), linear range (pg mL^{-1}), slopes of the weighted calibration curves ($\bar{x} \pm$ SD, $n = 3$), LOD and LOQ values.

	I SET-UP *LC-FS-HRMS*	II SET-UP *Direct injection LC-MRM-MS*	III SET-UP *Preconcentration LC-MRM-MS*
Retention time (min)[a]			
Ang I (1–10)	13.7 ± 0.08	7.9 ± 0.05	16.2 ± 0.04
Ang II (1–8)	12.9 ± 0.03	7.8 ± 0.04	15.8 ± 0.03
Ang-(1–7)	10.6 ± 0.04	6.4 ± 0.04	14.3 ± 0.06
Ang III (2–8)	12.5 ± 0.07	7.7 ± 0.06	15.7 ± 0.05
Ang IV (3–8)	13.1 ± 0.06	7.9 ± 0.05	16.1 ± 0.03
Precision (repeatability)[a]			
Ang I (1–10)	9.8	7.5	6.8
Ang II (1–8)	3.4	3.2	2.8
Ang-(1–7)	4.7	5.4	4.3
Ang III (2–8)	6.9	5.1	4.6
Ang IV (3–8)	4.1	4.4	3.1
Linear range (pg mL^{-1})	50–10,000	25–1,000	5–500
Slope			
Ang I (1–10)	0.06 ± 0.02[b]	0.0005 ± 0.0001[c]	0.0014 ± 0.0003[c]
Ang II (1–8)	0.66 ± 0.08	0.0024 ± 0.0003	0.0032 ± 0.0003
Ang-(1–7)	0.78 ± 0.10	0.0011 ± 0.0002	0.0018 ± 0.0002
Ang III (2–8)	0.05 ± 0.01	0.0004 ± 0.0001	0.0010 ± 0.0001
Ang IV (3–8)	2.34 ± 0.21	0.0015 ± 0.0002	0.0029 ± 0.0003
LOD (fg on column)[d]			
Ang I (1–10)	20	10	5
Ang II (1–8)	15	5	1
Ang-(1–7)	20	10	2
Ang III (2–8)	20	10	2
Ang IV (3–8)	15	5	2.5
LOQ (fg on column)[e]			
Ang I (1–10)	50	25	10
Ang II (1–8)	50	20	5
Ang-(1–7)	50	20	5
Ang III (2–8)	50	25	5
Ang IV (3–8)	40	15	7.5

Repeatability of injection, linearity and sensitivity of the methods were determined from analyses of calibration standards prepared in 1% (v/v) aqueous acetic acid solution. Repeatability of injection was estimated from five replicate analyses of calibration solutions at one concentration level and was expressed as RSD (%) of the peak areas of the corresponding peptides. Linearity of the methods was evaluated using calibration standards at six following concentration levels:
I set-up: 50 pg mL^{-1}; 200 pg mL^{-1}; 500 pg mL^{-1}; 1,000 pg mL^{-1}; 5,000 pg mL^{-1}; 10,000 pg mL^{-1} (IS–2,500 pg mL^{-1});
II set-up: 25 pg mL^{-1}; 50 pg mL^{-1}; 100 pg mL^{-1}; 250 pg mL^{-1}; 500 pg mL^{-1}; 1,000 pg mL^{-1} (IS–250 pg mL^{-1});
III set-up: 5 pg mL^{-1}; 10 pg mL^{-1}; 50 pg mL^{-1}; 100 pg mL^{-1}; 250 pg mL^{-1}; 500 pg mL^{-1} (IS–250 pg mL^{-1}).
[a] Estimated for peptide concentration: 200 pg mL^{-1} (set-up 1) and 100 pg mL^{-1} (set-up 2 and 3).
[b] ([Ring-D_5]Phe^8)-Ang II was used as an internal standard.
[c] [Asn^1, Val^5]-Ang II was used as an internal standard.
[d] Limit of detection (LOD) – (the smallest amount of analyte that can be distinguished from the background noise) was expressed as the amount for which the signal-to-noise ratio (S/N) was ≥3.
[e] Limit of quantification (LOQ) – the amount of analyte for which S/N ratio was ≥10.

3.2. Optimization of sample pretreatment

This study evaluated two different sample pretreatment methods: ultrafiltration and protein precipitation for analysis of Ang peptides in plasma samples. Three commercially available centrifugal ultrafiltration devices with different membrane orientation (flat-based or tangential) and chemistry were tested: *membrane A* – Microcon® YM-10, regenerated cellulose membrane (flat-based); *membrane B* – Roti®-Spin 10, polyethersulfone membrane (flat-based) and *membrane C* – Vivaspin® 5, polyethersulfone membrane (tangential). The angiotensin peptide process recovery results from the appropriate membrane devices A–C are presented on Fig. 2a. The obtained data indicate that the best peptide recoveries were achieved with the use of membrane B (Roti®-Spin, 10 kDa, PES) – recovery between 25.6% (for Ang I) and 56.7% (for Ang II). The worst recovery results were noticed for membrane C – the mean recovery for the peptides tested was at about 20%. These data strongly suggest that the specific membrane chemistry and orientation of the filter unit (influencing the transmembrane pressure and permeability of each membrane) determine the selectivity of proteins/peptides separation through the pores in the membrane. Additionally, peptide adsorption on the surface of sample tubes could contribute to its significant loss and poor recovery. However, although this approach is selective for low-molecular-weight peptides/proteins in a sample, the resulting filtrate may not allow for a thorough examination of the entire content of low-molecular-weight plasma proteome as the small proteins/peptides are commonly bound by large carrier proteins moderating their free and biologically active concentration in plasma.

On the other hand, ACN precipitation releases and therefore allows for analysis of higher number of small proteins/peptides attached to plasma albumin and other carrier proteins. Moreover, this approach has advantages over other sample preparation methods such as solid-phase extraction (SPE), liquid–liquid extraction (LLE), UF in that it is rapid, simple and allows a high process recovery by minimizing the possibility to loss of analytes during the sample clean-up process. To optimize this sample pretreatment approach, acetonitrile (neutral or acidified) with different dilution factors was evaluated. Optimal recoveries were obtained for blank

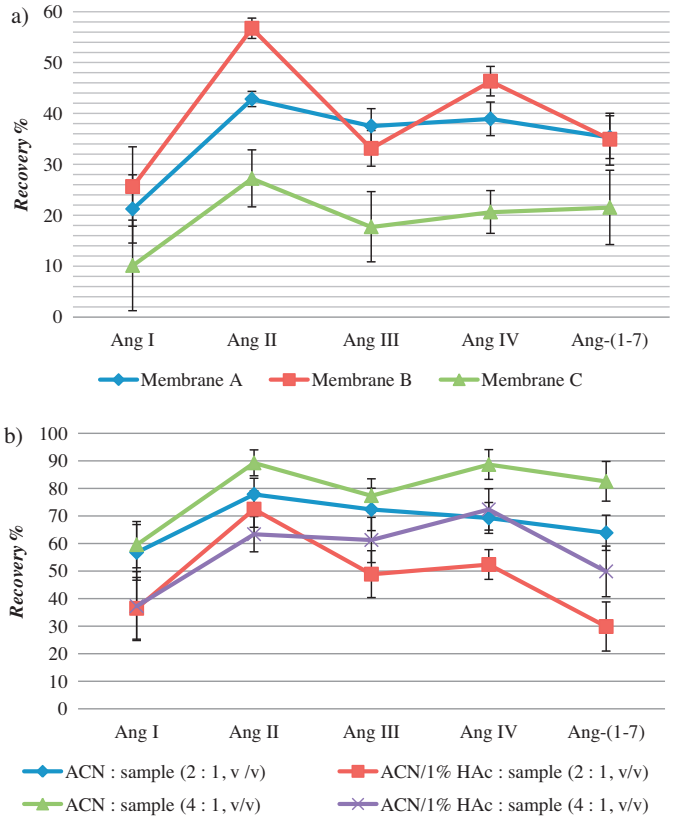

Fig. 2. Process recovery of angiotensin peptides from mouse plasma using various centrifugal ultrafiltration devices (a) and precipitation pretreatment (b). Values represent means ± SD ($n = 5$, for a concentration of peptides – 200 pg mL^{-1}). Membrane A – Microcon®, Millipore (10 kDa, regenerated cellulose (RC)); membrane B – Roti®-Spin, Carl Roth GmbH (10 kDa, polyethersulfone (PES)); membrane C – Vivaspin®, Sartorius (5 kDa, PES); HAc – acetic acid.

ACN (as protein precipitating solvent) with 4-fold dilution for plasma samples (Fig. 2b).

As the best recovery of the analytes from mouse plasma was achieved with the use of protein precipitation method, this sample pretreatment approach was used for further studies.

3.3. Evaluation of method performance

3.3.1. System suitability test

In order to check the overall performance of the nano-LC/MS system, system suitability was investigated with the use of PicoSure peptide mixture. This test was carried out to assess the absence of system dead volumes, reproducibility of the gradient delivery and analytical signal response. Representative chromatogram of the peptide mixture achieved with preconcentration nano-LC/MS system (set-up 3) is presented in Fig. S2. In the case where the repeatability in terms of retention times (RSD < 0.5%) and the area response (RSD < 1%) were maintained, the development and validation of the nano-LC–MS/MS method could be initiated.

3.3.2. Linearity and sensitivity of the method

All five calibration curves (constructed for each peptide) were linear over the concentration range of 5–500 pg mL^{-1} with correlation coefficient $R^2 \geq 0.99$. A straightline fit was made through the data points by least square regression analysis to provide the mean linear equations $y = ax + b$, where y was the peak area ratio of the peptide/IS ([Asn^1, Val^5]-Ang II) and x the concentration of the analyte. The LLOQ of the method was 5 pg mL^{-1}. The results of linearity (i.e., linear range, slope, Y-intercept) and the detection sensitivity

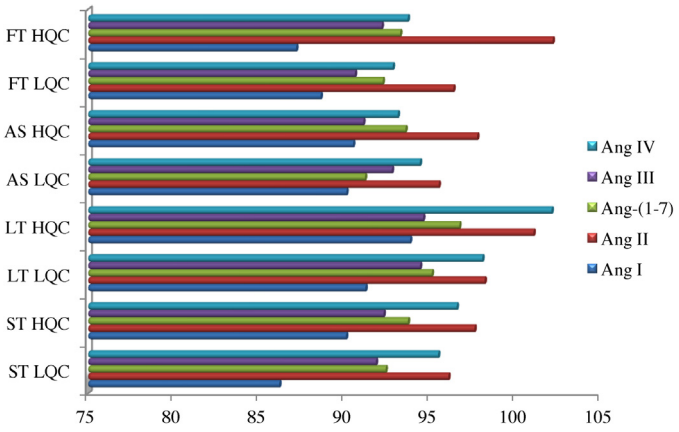

Fig. 3. Percent (%) of the nominal angiotensin peptide concentration after different storage conditions. Values are expressed as means, $n = 5$. LQC (low quality control) – 10 pg mL^{-1}; HQC (high quality control) – 400 pg mL^{-1}; ST – short-term temperature stability test (RT, 3 h); LT – long-term stability test (−80 °C for 1 month); AS – autosampler stability test (4 °C, 12 h); FT – freeze-thaw stability test (three cycles freezing-thawing).

(LLOQ) for discussed nanoflow LC-MS/MS method are presented in Table 4.

3.3.3. Accuracy, precision and process recovery

The intra-day and inter-day accuracy and precision were established from validation runs performed at three QC levels (Table 5). The accuracy was expressed as relative error (RE) of the QC samples, and the precision was demonstrated as relative standard deviation (RSD). For Ang II, the intra- and inter-day precision values (RSD %) were both not more than 5.1%, while the relative error (RE %) of assay accuracies ranged from −5.3 to 4.0%. For Ang-(1–7), the precision values were in the range 3.5–6.7%, while the RE of assay accuracies ranged from −7.5 to 6.3%. These results (as well as those obtained for the other peptides, Table 5) were within the acceptable criteria for accuracy and precision, demonstrating that this method was reliable and reproducible for the quantification of angiotensin peptides in mouse plasma samples.

The process recovery was estimated by comparing the peak area of the analyte obtained from the standard solution spiked in plasma followed by protein precipitation process to that of the analyte received from the matrix free solvent (directly injected from standard solution). In the case of analytes added before extraction, the peak area corresponding to the basal concentration of the analyte was deducted before the percent value of process recovery was calculated. The highest process recovery was observed for Ang II (89.9–91.9%), the lowest for Ang I (59.4–65.7%). There was no clear relationship between hydrophobicity of the analytes and recovery efficiency. Even, if the recovery of target analytes was not high, mainly due to the variable stability in biological matrices, the process recoveries were consistent and reproducible (Table 5) in all tested batches with insignificant variation (CV 7.2–11.9%).

3.3.4. Stability of the analytes

The stability tests were carried out by using low and high fortified QC samples. Fig. 3 summarizes the results of the short-term and long-term stability, autosampler stability and freeze-thaw stability of angiotensin peptides in mouse plasma as the mean percentages of the calculated vs. theoretical concentrations.

Many earlier studies highlighted very poor stability of Ang peptides in an *in vivo* environment, which is reflected by their short half-lives [21–23]. A significant loss of peptides was also reported in the standard stock solutions (consisting of a mixture of water/acetonitrile with a small amount of organic acids

Table 4
Calibration curves, correlation coefficients and linear ranges for angiotensin peptides in fortified plasma samples.

Analyte	Calibration curve	Correlation coefficient (R^2)	Linear range (pg mL^{-1})	LLOQ[a] (pg mL^{-1})
Ang I (1–10)	$y = 0.0010x + 0.1243$	0.9864	5–500	5
Ang II (1–8)	$y = 0.0029x + 0.2270$	0.9997	5–500	5
Ang-(1–7)	$y = 0.0015x + 0.1550$	0.9943	5–500	5
Ang III (2–8)	$y = 0.00085x + 0.1091$	0.9911	5–500	5
Ang IV (3–8)	$y = 0.0025x + 0.2325$	0.9985	5–500	5

[a] LLOQ was determined as the lowest concentration point of the standard curve, for which S/N ratio was ≥10 and RSD was ≤20%.

(formic/acetic)) [23]. The reasons for the disappearance of the analytes (in a stock solution) are not clear but could be due to binding to container walls. Angiotensin molecules can probably attach to various surfaces (plastic or glass vials, respectively) and become unavailable for detection. Therefore, to prevent losses of analytes, fresh standards for each analysis time were used, also the samples were extracted and analyzed immediately to shorten the sample preparation time.

Over a period of a short-term (3 h) stability at RT, a long-term (1 month) freezer stability at −80 °C, a freeze–thaw stability and 12 h injection time in the autosampler batch at 4 °C, the predicted concentrations for Ang peptides in QC samples deviated within 15% of the nominal values and no significant degradation products were observed in the samples. The results presented in Fig. 3 indicate that the new method for the determination of angiotensin peptides in mouse samples offers satisfactory stability, and is suitable for large-scale sample analysis.

3.3.5. Comparison with previously published methods

Several methods have been reported for analysis of angiotensin peptides in different biological matrices by LC/MS [22–25]. In the study of Elased et al. [24], surface-enhanced laser desorption/ionization time of flight mass spectrometry (SELDI-TOF-MS) with a ProteinChip Array technology (Ciphergen Biosystems, Inc., USA) was used for measurement of renin and ACE activity in plasma. The developed method was validated in animal models associated with alterations of the RAS confirming its usefulness for identification of novel substrates/inhibitors of the RAS. Some other time, Bujak-Gizycka et al. [22] used HPLC system (Merck, Germany) interfaced to a LCQ ion trap (Finnigan, USA), operating in selected ion monitoring (SIM) mode, for quantification of main products of angiotensin I metabolism *ex vivo* in various rat tissues and *in vitro* in culture of human endothelial cell line. The LLOQ obtained by the method based on this LC/MS configuration was 20 pM (100 attomole per injection) and was of the same order as those in immunoassays. Angiotensin peptide metabolism by endothelial cells (particularly, bovine adrenal microvascular endothelial cells (BMECs)) was also characterized by Cui et al. [23]. They have used HPLC system (Waters, USA) interfaced to a Micromass Quattro triple quadrupole (Waters) to monitor Ang II conversion/degradation by endothelial cells. The limit of quantification of the LC-MRM-MS method in a buffer matrix was estimated at 48 (Ang II) – 64 (Ang IV) femtomole range per injection. However, only the implementation of nanoLC combined with tandem mass spectrometry enabled to reach a significant increase in sensitivity corresponding to physiological concentrations of the endogenous Ang peptides. Hildebrand et al. [25] using triple quadrupole mass spectrometer (6430 Series, Agilent Technologies, USA) coupled to an HPLC-chip-system (Agilent) have studied the Ang I metabolism in human plasma at a low attomole level confirming the usefulness of their method for exploring the complex relationships within the RAS.

The method presented in this work has the best sensitivity compared with the other procedures, with a maximum loading at LLOQ per injection of 10 fg (8–13 attomole range). The significantly lower on-column loading of the analytes at LLOQ helps to maintain the column efficiency for many more injections. In contrast to other procedures, the method presented in this paper was fully validated, confirming its high analytical quality.

3.3.6. Quantification of angiotensin peptides in mouse plasma

The optimized LC/MS method was used to analyze angiotensin profile in plasma of wild type C57BL/6J and atherosclerotic ApoE/LDLR (−/−) mice.Fig. 4 presents representative LC/MS chromatograms of selected angiotensin peptides and internal standard from the plasma sample of ApoE/LDLR (−/−) mouse. ApoE and

Table 5
Intra- and inter-day precision, accuracy and process recovery of angiotensin peptides extracted from mouse plasma samples.

Analyte	Conc. [pg mL^{-1}]	Intra-day			Inter-day			Process recovery (mean ± SD, %)
		Measured (pg mL^{-1})	Accuracy (%)[a]	Precision (%)[b]	Measured (pg mL^{-1})	Accuracy (%)[a]	Precision (%)[b]	
Ang I (1–10)	10	8.8 ± 1.2	−12.1	13.9	8.6 ± 1.3	−14.5	15.3	59.4 ± 7.1
	250	217.9 ± 28.3	−12.8	13.0	215.9 ± 27.3	−13.6	12.7	
	400	361.0 ± 36.7	−9.8	10.2	354.0 ± 35.8	−11.5	10.1	65.7 ± 5.9
Ang II (1–8)	10	9.5 ± 0.5	−4.6	5.1	9.5 ± 0.4	−5.3	4.4	91.9 ± 6.6
	250	258.6 ± 6.9	3.5	2.7	258.4 ± 8.9	3.4	3.4	
	400	413.2 ± 12.5	3.3	3.0	415.9 ± 19.7	4.0	4.7	89.9 ± 6.8
Ang-(1–7)	10	10.5 ± 0.6	5.3	5.8	9.4 ± 0.6	−6.3	6.7	79.5 ± 7.3
	250	239.0 ± 10.2	−4.4	4.3	231.2 ± 11.5	−7.5	5.0	
	400	425.2 ± 14.7	6.3	3.5	375.7 ± 19.8	−6.1	5.3	81.3 ± 6.9
Ang III (2–8)	10	9.2 ± 0.8	−8.2	8.3	9.1 ± 0.9	−8.8	10.0	71.3 ± 7.4
	250	226.4 ± 20.3	−9.4	8.9	228.5 ± 21.3	−8.6	9.3	
	400	431.8 ± 22.6	7.9	5.2	438.3 ± 23.7	9.6	5.4	77.5 ± 6.9
Ang IV (3–8)	10	10.7 ± 0.7	6.8	6.1	9.5 ± 0.7	−4.9	7.7	85.6 ± 6.4
	250	263.9 ± 15.1	5.6	5.7	261.9 ± 14.7	4.8	5.6	
	400	380.7 ± 18.3	−4.8	4.8	421.7 ± 15.8	5.4	3.7	88.7 ± 6.6

[a] RE (relative error) (%) = [(measured value − theoretical value)/theoretical value] × 100.
[b] RSD (relative standard deviation) (%) = SD/average × 100.

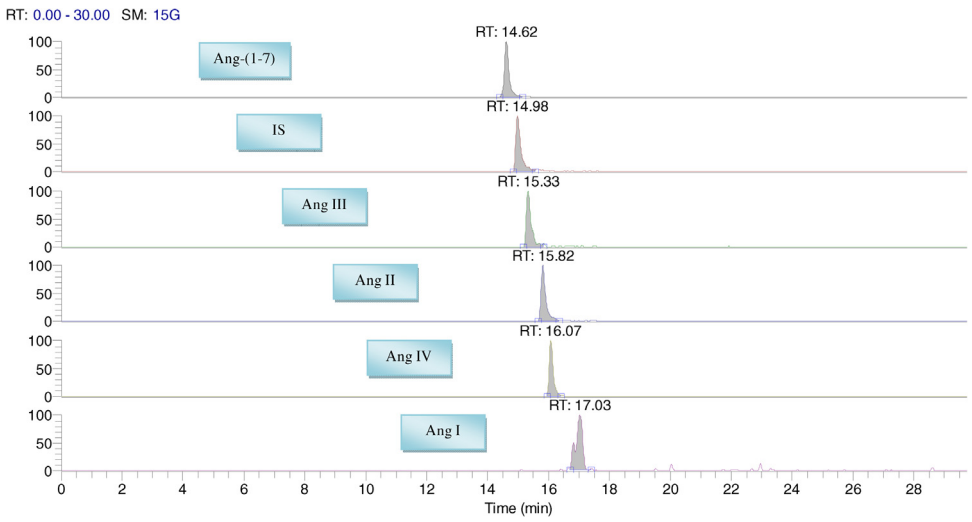

Fig. 4. Representative extract ion multiple reaction monitoring (MRM) chromatograms of angiotensin peptides and internal standard ([Asn^1, Val^5]-Ang II; 250 pg mL^{-1}) obtained by analysis of a selected (ApoE/LDLR (−/−)) un-spiked plasma sample using preconcentration nanoflow LC-MS/MS.

LDL receptor double knock-out mice are considered as one of the best animal model of atherosclerosis as they demonstrate severe hypercholesterolemia and develop advanced atherosclerotic lesions similar to human, that is preceded by development of endothelial dysfunction [26]. We chose this animal model as an object of study since many previous papers clearly indicate that the renin–angiotensin system with, in particular, its final product Ang II, plays a pivotal role in atherogenesis [13,27–30]. To our knowledge, we characterized for the first time, the endogenous angiotensin profile in these atherosclerotic mice.

We demonstrated that the major angiotensin peptides in plasma of normolipidemic mice were: Ang I and Ang IV (concentrations: 85.9 ± 44.9 and 122.2 ± 46.5 pg mL^{-1}, respectively). Atherogenesis was associated with a massive raise in angiotensin peptide concentration. This increase was most significant for Ang I (449.3 ± 81.4 pg mL^{-1}, approximately 5-fold increase as compared with WT), Ang II (200.2 ± 47.6 pg mL^{-1}, 5-fold increase) and Ang III (321.1 ± 44.2 pg mL^{-1}, 10-fold increase), but was less pronounced for Ang IV (412.4 ± 57.1 pg mL^{-1}, 3-fold increase). The concentration of Ang-(1–7) was not different between atherosclerotic and wild type mice (52.3 ± 11.4 vs. 56.2 ± 20.3 pg mL^{-1}, respectively), (Fig. 5). The results presented here are consistent with previous data, which indicate that hypercholesterolemia/atherosclerosis is associated with alterations in renin–angiotensin system that contributes to the development of cardiovascular diseases [13,31]. The observed pronounced increase in Ang I, Ang II, Ang III and Ang IV but not Ang-(1–7) suggests activation of renin as well as increase in ACE/ACE2 activity ratio. Our results appear to be consistent with the known pro-atherogenic role of renin [32,33] and ACE [34,35] as well as anti-atherogenic activity of ACE2 [36–38]. Which of the three culprits: renin, ACE or ACE2 is the major contributor to the development of atherogenesis, or whether they all play in concert, remains to be established. Surprisingly, despite the intense interest in the role of angiotensin peptides in atherogenesis, there are only few earlier studies attempted to describe the angiotensin distribution/concentrations in atherosclerotic mouse models [8,13,36].

While method presented here was optimized and validated for analysis of mouse plasma, we believe that it could be quickly adopted for evaluation of angiotensin profile in human plasma for clinical applications such as diagnosis of the RAS abnormalities or monitoring of the RAS inhibition-based therapies. Angiotensin peptide concentrations in human blood are in the same range as in mouse blood [25,39]. Studies on function of the RAS are rapidly progressing and its new components are being constantly

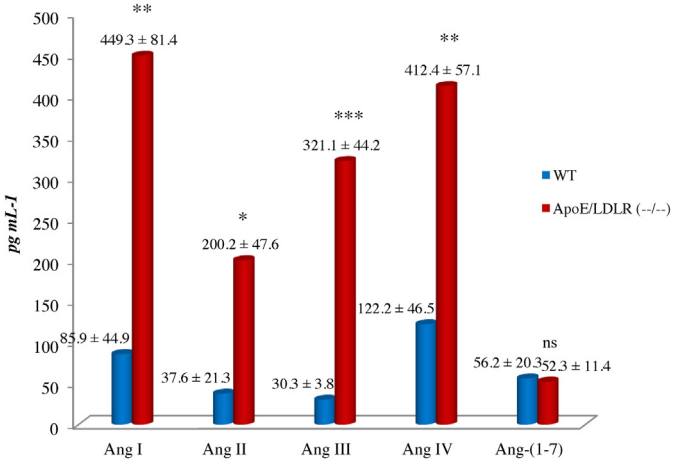

Fig. 5. Plasma concentrations of angiotensin peptides in WT (n = 5) and ApoE/LDLR (−/−) (n = 8) mice (a recovery correction was not applied). Data are represented as mean ± SEM. *P < 0.05, **P < 0.01, ***P < 0.001, ns – not significant vs. WT group. ApoE/LDLR (−/−) – apolipoprotein E/LDL receptor double knock-out (mouse); WT – wild type (mouse).

identified. This includes peptides such as Ang-(1–9), Ang A or alamandine [40–42]. While their function and need for routine monitoring require further confirmation, our method, as any MS based procedure could be easy expanded to detect also these newly discovered members of the RA system.

4. Conclusions

We have presented an optimal methodology for the extraction, isolation and quantification of angiotensin peptides in mouse plasma. The use of tandem mass spectrometry in combination with nanochromatography and a new generation nanoESI ion source provided a significant gain in sensitivity with excellent accuracy and precision. Consequently, the method proposed is substantially advanced as compared with previously published methodologies. The developed procedure was successfully used to measure the angiotensin peptide concentrations in mouse plasma highlighting for the first time substantial elevation of concentration of Ang I, Ang II, Ang III and Ang IV, but not Ang-(1–7) in atherosclerotic ApoE/LDLR (−/−) mice as compared to wild types. Taking into consideration the important role of the RAS in cardiovascular diseases,

the proposed methodology could be a useful tool for monitoring the therapeutics effects of various treatment schemes in atherosclerosis and heart failure. Furthermore, angiotensin pathways profiling could provide better insight into alterations of ACE/ACE2 homeostasis in the development of endothelial dysfunction and vascular inflammation.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.chroma.2015.03.012.

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