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RESEARCH PAPER

Impurity identification and determination for the peptide hormone angiotensin I by liquid chromatography–high-resolution tandem mass spectrometry and the metrological impact on value assignments by amino acid analysis

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Abstract It is common practice to quantify the mass concentration of a peptide solution through quantitative determination of selected chemically stable amino acids produced following complete hydrolysis of the parent peptide. This is because there is generally an insufficient quantity of material available to allow for the obvious alternative of a direct purity analysis characterization of the parent peptide, and the subsequent constitution of a calibration solution. However, selected accurately characterized pure peptide reference materials are required to establish reference points for the dissemination of metrologically traceable measurements and to develop reference measurement systems for laboratory medicine. In principle, purity assignment of a peptide can be performed by using the so-called mass balance approach, by employing a range of analytical techniques to obtain an estimate of the mass fraction content of all impurities present in the intact peptide, and by utilizing the difference from the theoretical limit value to assign the mass fraction content of the main peptide. Liquid chromatography–high-resolution tandem mass spectrometry (LC-hrMS/MS) is a key technique for the detection, identification, and determination of structurally related impurities present in a peptide material, and experiments characterizing the model peptide hormone angiotensin I (ANG I) are described in the present work. Degradation products that were generated from ANG I after storage at elevated temperatures were screened. The formation of peptide fragments such as ANG II or ANG III was determined by comparison of measured mass values with calculated mass values. The use of a data-dependent acquisition technique enabled the detection and structural characterization of ANG II and other peptide fragments as major impurities in the same LC-hrMS/MS analysis run. Subsequent quantification using external calibration allowed the mass fraction of the major impurities in a candidate reference material to be estimated as 10.4 mg/g. Failure to correct for these impurities would lead to a 1 % error in the determination of the concentration of the peptide in solution by amino acid analysis techniques.

Keywords Angiotensin I · Peptide · Impurities · Liquid chromatography · High-resolution mass spectrometry

Introduction

The peptide prohormone angiotensin I (ANG I) is an essential component of the renin–angiotensin system (RAS), also referred to as the renin–angiotensin–aldosterone system (RAAS), which is involved in the regulation of the water and salt balance and blood pressure in the human body, but also has a significant influence on the cardiovascular, renal and metabolic systems [1, 2]. The linear decapeptide ANG I is formed by enzymatic cleavage of the protein angiotensinogen, which is produced in the liver. ANG I is composed of ten amino acids (Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu) and

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is further converted to the octapeptide angiotensin II (ANG II), the main effector of the RAS [3]. Among other effects, ANG II is involved in cardiovascular homeostasis, the control of cellular growth, and the release of the steroid hormone aldosterone from the adrenal gland [4]. Enzymatic cleavages of N- and/or C-terminal amino acids from ANG II result in the formation of smaller peptides, which have various effects in the RAS, such as ANG III, ANG IV, and ANG (1–7) [5–8].

Taking the significant medical importance of angiotensin peptides into consideration, the literature contains numerous studies covering the development and application of methods of analysis for ANG I and related fragment peptides based on mass spectrometry, in most cases combined with prior separation of substances by liquid chromatography. These methods include screening for ANG metabolites using LC-MS in MS full-scan mode [5], studies of electrospray ionization—(tandem) mass spectrometric (ESI-MS(/MS)) properties of ANG II and analogous compounds [9], and sensitive LC-MS/MS methods in the selective reaction monitoring (SRM) mode for the simultaneous quantification of chosen ANG peptides [4, 10].

Accurate diagnosis, monitoring, and treatment are key areas of healthcare systems that require reliable and comparable measurements. Typically, in contrast to the thorough purity analyses that can be undertaken directly on small organic compounds, it is common practice in the field of peptide analysis—due to an insufficient amount of the parent peptide to allow direct quantification and eventually difficulties in handling—to instead quantify the mass fraction of a peptide solution by amino acid analysis through the quantitative determination of selected amino acids after complete hydrolysis. The performance of this amino acid analysis methodology has been studied in pilot studies for peptide calibrators within the Bioanalytical Working Group (BAWG) of the Consultative Committee for Amount of Substance (CCQM). Highly repeatable and accurate hydrolysis methods and the gravimetric preparation of stable solutions of both peptide and reference amino acids of known purity with corresponding uncertainty are required [11–13]. However, there is a possibility that peptide mass fractions or purity values assigned by amino acid analysis are significantly overestimated, in particular when potential peptide impurities contain the same number of selected amino acids as the model peptide. Therefore, sufficiently directly characterized pure peptide reference materials and suitable methods for their comprehensive characterization are still required for the dissemination of metrologically traceable measurements and to develop reference measurement systems (RMS) for laboratory medicine as outlined in ISO 17511 [14]. In general, purity is assigned using the mass balance approach. The mass fraction of the various impurities present in the material is estimated and, by subtraction, a measure of the mass fraction content of the main component of the material is obtained [15, 16]. Typically, a range of analytical techniques (GC-MS, GC-FID, LC-MS, LC-UV, etc.) are used to obtain an estimate of the mass fraction content of the intact peptide and also to provide information on its qualitative composition and the nature of any related structure impurities.

Liquid chromatography (LC) coupled to high-resolution tandem mass spectrometry (hrMS/MS) is one of the key techniques for determining related structure impurities in peptides. Improvements in modern measurement and mass spectrometric techniques allow for more comprehensive characterization and quantification of larger molecules, such as peptides and small proteins, thus enabling the development of an RMS for these important analytes. Full characterization and quantification can probably only be achieved by using the abovementioned mass balance approach and/or through a combination of several complementary analytical methods; for example, the use of a specific method for the determination of single free amino acids that would typically not be detected by a specific peptide method.

In this paper, we report on the characterization of an analytical candidate reference material for ANG I using LC-hrMS/MS and the potential metrological impact on value assignments by amino acid analysis. The focus is on the qualitative and quantitative analysis of ANG I and structurally related peptides, with the aim being to assess the purity of a candidate reference material. Artificial alteration of an ANG I material will also be discussed to simulate degradation of the analyte substance. The metrological impact on value assignments by amino acid analysis is discussed on the basis of these results.

Materials and methods

Chemicals and standards

Acetonitrile (HiPerSolv Chromanorm, HPLC gradient grade) and formic acid (98 % purity) were obtained from VWR (Fontenay-sous-Bois, France) and hydrochloric acid from Sigma Aldrich (Lyon, France). Deionized water was obtained from a Milli-Q gradient system from Millipore (Molsheim, France) and used for the preparation of solutions.

The human peptide angiotensin I (ANG I, sequence DRVYIHPFHL, trifluoroacetate salt) was purchased as a synthetic standard compound from Chemos GmbH (Regenstauf, Germany). The human peptides angiotensin II (ANG II, DRVYIHPF), angiotensin III (ANG III, RVYIHPF), angiotensin IV (ANG IV, VYIHPF), angiotensin (1–4) (ANG (1–4), DRVY), and angiotensin (4–8) (ANG (4–8), YIHPF) were acquired as synthetic standard compounds from GenScript USA Inc. (Piscataway

Township, NJ, USA). Two candidate materials (internal IDs: OGO.096b and OGO.096c) of human angiotensin I (ANG I) were provided by the National Institute of Standards and Technology (NIST, Gaithersburg, MD, USA).

Preparation of standards

For structural characterization of the angiotensin peptides by infusion-hrMS/MS and to develop an LC-hrMS/MS method, single standard solutions of the peptides were prepared gravimetrically, using 0.1 N hydrochloric acid as a solvent, with the following mass fractions (stated with combined uncertainties): ANG I: 95.181 μg/g±0.005 μg/g, ANG II: 103.037 μg/g± 0.005 μg/g, ANG III: 97.395 μg/g±0.005 μg/g, ANG IV: 93.718 μg/g±0.005 μg/g, ANG (1–4): 98.087 μg/g± 0.005 μg/g, and ANG (4–8): 96.284 μg/g±0.005 μg/g. The solutions were prepared gravimetrically using 0.1 N hydrochloric acid because they were used for the development of other analytical methods as well. Standard solutions were prepared gravimetrically from human angiotensin I (ANG I), using purified water (Milli-Q gradient, Millipore) as a solvent and the following mass fractions (including combined uncertainties): Batch ID OGO.096b: 592.599 μg/g±0.030 μg/g, ID OGO.096c: 587.606 μg/g±0.030 μg/g.

For direct infusion MS(/MS) analysis, the single standards were diluted to a working mass concentration in the range of 1 mg/L to 11 mg/L, using an aqueous solvent consisting of water (Milli-Q)+acetonitrile (1+1, v/v) and 0.1 % formic acid (v/v).

For LC-hrMS(/MS) measurements, the standard solutions were diluted to working mass concentrations in the range of 7.6 μg/L to 108.6 mg/L, using an aqueous solvent consisting of water (Milli-Q)+acetonitrile (1+1, v/v) and 0.1 % formic acid (v/v), depending on the method parameters used (cut-off of chromatographic peak of ANG I at higher analyte concentrations, see below).

Hamilton glass syringes with stainless steel plungers and laboratory glassware were used to manipulate the standard solutions in order to avoid contaminating the mass spectra with ion signals from synthetic polymers arising from plastic pipette tips and plastic tubes.

Heat degradation experiments

Heat degradation experiments were undertaken to study if the predominant impurities in ANG I are degradation fragments of ANG I produced by the sequential loss of amino acids. To artificially create degradation products, aliquots of a solution prepared using the ANG I material from Chemos were exposed to high temperatures and long storage periods. In preliminary experiments, the following conditions were found to accelerate the degradation: an ANG I solution of a mass concentration of 10.9 mg/L, prepared with an aqueous solvent consisting of water (Milli-Q)+acetonitrile (1+1, v/v) and 0.1 % formic acid (v/v), was stored at 60 °C in a water bath for up to 29 h, with the temperature monitored using a thermometer. The same volumetric flask containing ANG I was then stored at 90 °C in a drying oven for up to 143 h. During the storage, aliquots were taken from the solution at different times (e.g., 87 h after the start of storage at 90 °C), transferred into LC vials, and subjected to LC-hrMS/MS experiments.

Instrumentation

An LC series 1200 instrument (Agilent, Waldbronn, Germany) consisting of a model G1379B degasser, G1312B binary pump SL, G1367C autosampler SL, and G1316B column thermostat SL was employed for LC-hrMS/MS analysis. The LC was coupled online to an LTQ-Orbitrap XL high-resolution tandem mass spectrometer (Thermo Electron, Bremen, Germany) fitted with an electrospray ionization (ESI) source.

LC conditions

Chromatographic separation was performed on a Jupiter Proteo column, 150 mm×2 mm, 4 μm, 90 Å (Phenomenex, Le Pecq, France). After comprehensive optimization, the following conditions were used for all subsequent LC-hrMS/MS experiments with angiotensin standard solutions: flow rate was 0.4 mL/min, the column thermostat temperature was 22 °C, the autosampler rack temperature was 5 °C, and the injection volume was 10 μL. Eluent A consisted of water and 0.1 % formic acid (v/v) and eluent B was acetonitrile with 0.1 % formic acid (v/v). The LC gradient programme was as follows: 3 % eluent B (0.5 min hold), gradient to 17.5 % eluent B in 40.5 min, gradient to 95 % B in 2 min (8 min hold), back to 3 % B in 2 min, and hold for 9 min (total method duration: 62 min).

In preliminary LC-hrMS experiments, including a MS full scan from *m*/*z* 100 to *m*/*z* 2000 in the FTMS analyzer (Orbitrap) at a mass resolution of 30,000 [FWHM (full width at half maximum) at *m*/*z* 400], the retention time (RT) of ANG I (35.6 min) was determined to define a "cut-off" period for the subsequent characterization of impurities in highly concentrated ANG I standard solutions. The LC effluent was bypassed to waste before entering the ESI source using an automatic valve connected upstream for the time period of the ANG I elution from the column. This prevented the MS from being contaminated in the case of measurements of highly concentrated standard solutions.

Depending on the retention time and shape of the ANG I peak, the cut-off period for ANG I was set to an appropriate interval, e.g., 34.80–36.80 min for the analysis of the candidate ANG I material. This period was determined at the beginning of each measurement series by using a standard solution at a mass concentration of about 12 mg/L, and was

subsequently implemented in the methods used to analyze standard solutions of the ANG I candidate material at a mass concentration of about 100 mg/L.

General instrument parameters for the mass spectrometer

General mass spectrometer settings for both direct infusion MS experiments and LC-hrMS/MS experiments: ESI probe position: D (vertical), middle (left–right), 2 (forward–backward), source voltage: 4000 V, positive ion mode, isolation width for MS/MS precursors: 2, analyzer fill time settings: ion trap full max ion time: 200 ms, ion trap MS^n max ion time: 100 ms, FTMS full max ion time: 500 ms, FTMS MS^n max ion time: 2000 ms, 1 microscan was included in all scan modes. Sheath gas: 90 arb (arbitrary units), auxiliary gas: 5 arb, sweep gas: 0 arb, capillary temperature: 370 °C.

Direct infusion MS experiments only: size of Hamilton glass syringes for direct infusion: 500 μL, flow rate for direct infusion: 10 μL/min.

Optimization of MS parameters for angiotensin peptides

In a preliminary LC-MS run, the retention times of the individual angiotensin peptides, which were available as pure standards, were determined (data not shown). For the subsequent optimization of MS parameters for the individual peptides, the composition of the LC eluent was adjusted accordingly. The acetonitrile concentration was increased from 3.6 % to 14.8 % (v/v) in water, depending on the solvent composition at the retention time of the respective peptide, for which the optimum instrument conditions were determined by the tuning procedure. The peptide standard solutions of the angiotensin peptides ANG I (Chemos) and ANG II, ANG III, ANG IV, ANG (1–4), and ANG (4–8) (GenScript), which were intended for infusion, were diluted with the same content of formic acid (0.1 % (v/v) and acetonitrile (3.6 %–14.8 % (v/v)) as the LC eluent.

To obtain optimal signal intensities for the angiotensin peptides, the LTQ mass analyzer was tuned using diluted single standard solutions at mass concentrations of 10 μg peptide/mL. The peptides were infused at a flow rate of 10 μL/min using a T-piece into an LC eluent (aqueous acetonitrile +0.1 % formic acid (v/v)) at a flow rate of 0.4 mL/min. An individual tuning file containing the optimized instrument settings was created for each of the angiotensin-related peptides. For the evaluation, all tuning files were implemented in LC-hrMS/MS test methods, which were used to analyze a peptide standard mixture containing 0.4 mg/L of each of the abovementioned peptides. The tuning file for the $[M+2H]^{2+}$ ion of ANG II provided the best signal intensities for MS full-scan peaks of the respective peptides, and was therefore implemented in all subsequent LC-hrMS/MS data acquisition methods.

Development of a data-dependent LC-hrMS/MS method

A data-dependent (DD) LC-hrMS/MS method was set up in the "10th Order Double Play" mode of the LTQ-Orbitrap XL and optimized for the individual peptides. This consisted of the following analysis events. Initially, a full MS scan was performed from *m/z* 100 to *m/z* 2000 in FTMS mode at a mass resolving power of 30,000 (FWHM) (scan event 1, survey scan). The ten most intense ions observed in the survey scan, with intensities of at least 1000 cps (peak heights), were subjected to subsequent MS/MS scans (scan event 2) in the LTQ mass analyzer.

A positive precursor ion mass list (referred to as "Parent Mass List" in the Xcalibur software) was predefined that contained calculated theoretical monoisotopic masses of $[M+H]^+$, $[M+2H]^{2+}$ and $[M+3H]^{3+}$ ions of 45 possible ANG I-related peptides ranging in length from dipeptides to the decapeptide ANG I and the dodecapeptide angiotensinogen (based on observations in preliminary LC-hrMS experiments). The comprehensive precursor ion mass list is provided in Table S1 of the "Electronic supplementary material" (ESM). Providing the selected precursor ions appeared in the survey scan with signal intensities of at least 1000 cps (peak heights), they were considered for subsequent MS/MS scans.

Likewise, a negative precursor "Reject Mass List" was created for ions that should be ignored for MS/MS scans; this is also provided in Table S1. The list contains precursor ions in the range from *m/z* 107 to *m/z* 759, with intensities of at least 10000 cps (peak heights), that were obtained from mass spectra recorded from solvent blank runs (e.g., solvent impurities).

The counts per second (cps) values mentioned are conservative threshold values for further data acquisition that were established based on preliminary experiments to initially separate the wheat from the chaff. The cps values are instrument dependent while the corresponding mass concentrations are strongly analyte dependent. 1000 cps and 10,000 cps in relation to the major analyte ANG I would correspond to about 0.05 mg/L (0.5 pg absolute) ANG I and 0.5 mg/L (5 pg absolute) ANG I, respectively. 1000 cps and 10,000 cps in relation to the least abundant impurity ANG (4–8) would correspond to about 1.05 mg/L (10.5 pg absolute) ANG (4–8) and 10.5 mg/L (105 pg absolute) ANG (4–8), respectively.

The values of the masses entered in the negative list differed by at least±*m/z* 0.2 from the values of the selected peptides.

The DD criteria were as follows: activation type, collision-induced dissociation (CID); isolation width, 2; collision energy, 35; default charge state, 2; activation Q, 0.25; and activation time, 30 ms. The following options were enabled: most intense if no parent masses found, FT master scan preview mode, charge state screening, and rejection of unassigned charge states.

Global DD settings: exclusion mass width by mass, low 0.5/high 2.5; parent mass width and reject mass width relative to mass, low 5 ppm/high 5 ppm; repeat count, 2; repeat duration, 20 s; exclusion list size, 500; and exclusion duration, 20 s.

Characterization of different ANG I materials by LC-MS/MS

Aliquots of a commercially available ANG I (Chemos) were dissolved and analyzed at a mass concentration of 109 mg/L using an optimized DD LC-hrMS/MS method (described in the previous section) in which the cut-off range for the ANG I peak was set at RT from 35.35 min to 36.50 min. Standard solutions from a heat degradation experiment (storage of the peptide at 4 °C and 90 °C) were compared with the aim of determining a qualitative description of the peptide-related impurities potentially formed. In addition, both of the ANG I candidate materials (ID OGO.096b, OGO.096c) were characterized (via three replicates) using the same DD LC-hrMS/MS method, but with an optimized cut-off range for the ANG I peak.

To derive a semi-quantitative estimation of the mass concentrations of major peptide-related impurities in the candidate ANG I materials, external calibration experiments were carried out. A peptide standard solution mixture was prepared, containing 16.7 mg/L each of ANG I, ANG II, ANG III, ANG IV, ANG (1–4) and ANG (4–8), by combining 200 μL of each peptide standard in a common LC vial. Five standard levels of this peptide mixture were prepared in three replicates, with mass concentration levels of 8.3 μg/L, 55.5 μg/L, 111.1 μg/L, 166.7 μg/L, and 833.3 μg/L per peptide. For all dilutions, a solvent consisting of water + acetonitrile (97+3, v/v) and 0.1 % formic acid (v/v) was used. All standard levels were measured using a similar LC-hrMS/MS method, lacking the cut-off of the major ANG I peak. To obtain an indication of the intermediate precision, the entire analyses of the ANG I candidate materials were repeated on a separate day.

Evaluation of the LC-hrMS/MS data

The measurement data were analyzed using the manufacturer's Xcalibur software (version 2.1), which was supplied with the mass spectrometer. To identify peptides, theoretical MS/MS fragment ions [17, 18] of ANG I and probable related peptides were calculated using the website MS-Product [19] and compared with the experimental MS/MS data. Peak areas and heights for selected molecular ions of the peptides were determined and used for the semi-quantitative estimation of their mass concentrations.

Results and discussion

Optimization of LC conditions and characterization of ANG I by LC-MS/MS

ANG I and the related peptide fragments ANG II, ANG III, ANG IV, ANG (1–4), and ANG (4–8) were selected and ordered as synthetic standards from commercial suppliers, based on results from preliminary infusion MS/MS and LC-MS/MS measurements and information from the literature. The synthetic standards were initially used to optimize chromatographic separation conditions for the angiotensin peptide fragments. Figure 1 shows an LC-hrMS run with MS full-scan experiments recorded using the Orbitrap mass analyzer at high mass-resolving power. It shows an overlay of extracted ion current chromatograms of the most intense ion species of the peptides, with a mass accuracy of ±5 ppm for the specified *m*/*z* values.

The optimized method resulted in a baseline separation of the peptide peaks and the elution of ANG I as the last component. The elution of ANG I as the last component was anticipated because the structurally related impurities of interest are shorter fragments and are therefore expected to elute before ANG I. Due to its chemical properties, the peptide fragment ANG (1–4) eluted much sooner than the other selected peptides. However, it was impossible to achieve a longer retention for ANG (1–4) without obtaining much longer retention and undesired peak broadening for the other peptides.

Subsequently, the electrospray ionization (ESI) parameters were optimized for the abovementioned six peptides. The ESI parameters were optimized in preliminary LC-MS runs. Those resulting in the most intense signals for the peptides were selected for the implementation of the corresponding instrument tuning files in the LC-hrMS/MS measurement methods.

LC-hrMS/MS analysis of the impurities of a commercial ANG I material

After optimizing the LC and ESI conditions for ANG I and selected related peptides, a data-dependent (DD) LC-hrMS/MS method was created. This method enabled automatic recording of MS/MS product ion scans of candidate molecular ions that had been detected in preceding MS full-scan experiments conducted at high mass-resolving (hr) power in the same analysis run. Selected candidate ions showed an *m*/*z* value close to the theoretical monoisotopic *m*/*z* of possible shortened peptides formed from ANG I in a degradation process enhanced by storage at high temperature. Omnipresent contaminants that had been detected in preceding LC-hrMS experiments were filtered using an exclusion mass list in subsequent measurement methods.

This DD LC-hrMS/MS method was further optimized and used to analyze two aliquots of a solution of a commercially

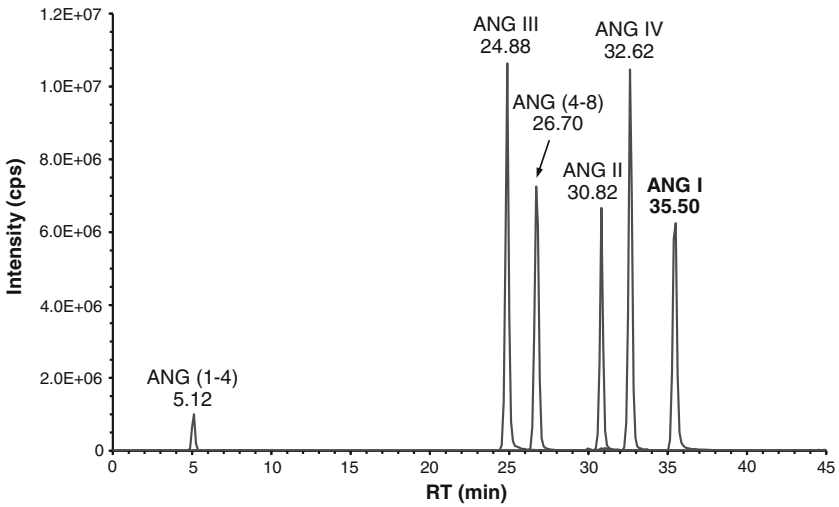

Fig. 1 LC-hrMS of a standard mixture containing six ANG peptides: overlay of extracted ion current chromatograms of *m/z* 552.27764 (ANG (1–4)), *m/z* 466.26106 (ANG III), *m/z* 338.67630 (ANG (4–8)), *m/z* 523.77453 (ANG II), *m/z* 388.21051 (ANG IV), and *m/z* 432.89977 (ANG I). Mass tolerance was±5 ppm

available ANG I material at a mass concentration of 109 mg/L, which were stored at 4 °C and 90 °C for 143 h, respectively. Several shortened peptides formed by the cleavage of one or more amino- or carboxy-terminal amino acids from the ANG I were predominantly detected (Table 1). The ANG I material was also characterized by direct infusion MS/MS experiments with heat-degraded aliquots (data not shown).

Consecutive loss of N- or/and C-terminal amino acids at the ANG I molecule was observed during storage at high temperature (e.g., at 90 °C) for time periods of up to a couple of days. Such harsh thermal conditions had an adverse effect on the chemical stability of the decapeptide ANG I. These results suggested the possibility of finding similar structurally related impurities in ANG I candidate material analyzed at high concentrations.

Qualitative LC-hrMS/MS analysis of impurities in ANG I candidate materials

Subsequently, two ANG I candidate materials were subjected to analysis using the same DD LC-hrMS/MS method. Mass fractions of 98.77 μg/g and 97.93 μg/g were injected for

Table 1 ANG I-related peptide fragments occurring in a commercially available ANG I material after storage at 4 °C and 90 °C for 143 h, as analyzed by LC-hrMS/MS. Criteria: proof by standard or intensity ≥5.0×10^4 cps

Occurring ANG peptide (impurity)	Amino acid sequence (1-letter code)	RT (min)	Charge state	Measured *m/z*	Calculated *m/z*	Mass deviation (ppm)	Peak height (cps)	MS/MS spectrum available
Storage temperature: 4 °C (control)								
ANG (2–10)	RVYIHPFHL	30.50	3	394.55713	394.55746	−0.836	1.06×10^6	Y
ANG II	DRVYIHPF	31.13	2	523.77441	523.77453	−0.229	8.76×10^5	Y
ANG (4–10)	YIHPFHL	31.77	3	309.50012	309.50095	−2.682	2.06×10^5	Y
ANG (1–9)	DRVYIHPFH	22.48	3	395.20413	395.20508	−2.404	1.96×10^5	Y
ANG (1–6)	DRVYIH	8.36	2	401.71384	401.71394	−0.249	1.19×10^5	Y
ANG III	RVYIHPF	25.23	2	466.26059	466.26106	−1.008	1.57×10^4	N
Storage temperature: 90 °C								
ANG (2–10)	RVYIHPFHL	30.22	3	394.55618	394.55746	−3.244	9.01×10^7	Y
ANG III	RVYIHPF	25.12	2	466.26056	466.26106	−1.072	8.28×10^5	Y
ANG (8–10)	FHL	19.69	2	208.61772	208.61825	−2.541	4.11×10^5	N
ANG (3–10)	VYIHPFHL	23.80	1	1025.55615	1025.55671	−0.546	3.13×10^5	N
ANG (2–5)	RVYI	14.45	1	550.33441	550.33476	−0.636	1.05×10^5	N
ANG (1–3)	DRV	1.35	1	389.21384	389.21431	−1.208	1.04×10^5	N
ANG IV	VYIHPF	32.87	2	388.20996	388.21051	−1.417	5.14×10^4	N
ANG II	DRVYIHPF	30.96	2	523.77368	523.77453	−1.623	4.37×10^4	Y
ANG (1–4)	DRVY	8.20	2	276.64185	276.64246	−2.205	2.43×10^4	Y

RT retention time, *ppm* parts per million, *cps* counts per second

OGO.096b OGO.096c, respectively. The focus of the evaluation was the determination of possible shortened peptides generated by the gradual degradation of ANG I during storage, as observed during the heat storage experiments. Additionally, external calibrations were performed with the six synthetic peptides ANG I, ANG II, ANG III, ANG IV, ANG (1–4), and ANG (4–8) in a defined mass concentration range. All measurements were performed in two different series (different days) with three replicates each. Figure 2 shows the LC-hrMS/MS characterization of the peptide ANG (2–10), which occurred as a significant impurity in the ANG I standard OGO.096b.

The triply charged molecular ion of ANG (2–10) was found to be the most abundant ion. It is shown as an extracted ion trace in the extracted ion current chromatogram (Fig. 2a). The LC effluent was not analyzed by the mass spectrometer during the time period 34.8–36.8 min in order to prevent contamination of the instrument with the major compound ANG I, which was injected at a high concentration. Underlying the LC peak at RT 30.21 min, diagnostic ions of the charge states +1 to +4 of ANG (2–10) appeared in the hrMS full-scan mass spectrum (Fig. 2b). To determine the primary structure (amino acid sequence) of this peptide, the triply charged molecular ion (*m/z* 394.56) was selected as the precursor and fragmented by CID collision in the LTQ analyzer of the instrument. This resulted in a meaningful product ion series of b- and y-ions [17, 18], confirming the peptide sequence RVYIHPFHL (Fig. 2c). When comparing the *m/z* values of experimentally obtained fragment ions with those calculated using the website MS-Product, good agreement was achieved. MS/MS scans of the other charge states observed for ANG (2–10), such as singly and doubly charged molecular ions, also yielded informative product ion spectra, but at lower signal intensities. Table 2 gives an overview of the detected ANG I-related peptides for which MS/MS spectra were generated, in order of decreasing amino acid chain length. Most of the detected peptides had been degraded at the N-terminus; some peptides also missed up to four amino acids at the C-terminus.

Quantification of selected peptide impurities in the ANG I candidate materials (OGO.096b and OGO.096c)

Original standards for the peptides ANG I, ANG II, ANG III, ANG IV, ANG (1–4), and ANG (4–8) were used for external calibration experiments in the mass concentration

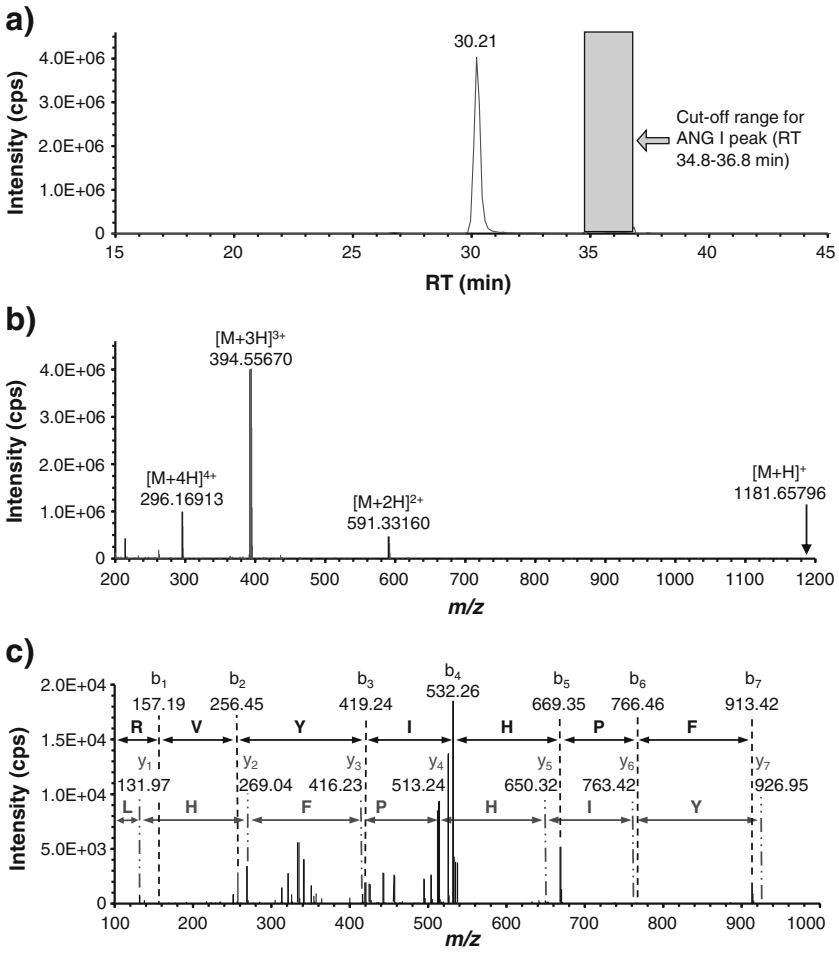

Fig. 2 *a–c* Extracted ion current chromatogram of the $[M+3H]^{3+}$ ion of ANG (2–10) at *m/z* 394.55746, occurring as an impurity in an ANG I standard injected into LC-hrMS/MS at high concentration (**a**). hrMS spectrum with four charge states of ANG (2–10) underlying the LC peak at RT 30.21 min (**b**). MS/MS spectrum of ANG (2–10) at the same RT, showing the peptide sequence RVYIHPFHL (**c**)

Table 2 Overview of ANG I and related peptide fragments in two candidate materials, as detected and confirmed by LC-hrMS/MS.

Occurring ANG peptide (impurity)	AA chain length	AA sequence	MW (g/mol)	RT (min)	
				OGO.096b	OGO.096c
ANG I	10	DRVYIHPFHL	1295.8	35.76	36.09
ANG (1–9)	9	DRVYIHPFH	1182.6	22.60	22.53
ANG (2–10)	9	RVYIHPFHL	1180.7	30.69	30.62
ANG II	8	DRVYIHPF	1045.5	31.31	31.19
ANG (3–10)	8	VYIHPFHL	1024.6	–	33.73
ANG III	7	RVYIHPF	930.5	–	25.27
ANG (4–10)	7	YIHPFHL	925.5	31.87	31.93
ANG (1–6)	6	DRVYIH	801.4	8.40	8.34
ANG IV	6	VYIHPF	774.4	–	32.99
ANG (5–10)	6	IHPFHL	762.4	25.47	25.39
ANG (6–10)	5	HPFHL	649.3	–	34.27
ANG (7–10)	4	PFHL	512.3	23.58	23.90
ANG (8–10)	3	FHL	415.2	19.81	–
ANG (7–8)	2	PF	262.1	–	31.19

Underlined substances were available as original standards

range 8.3–833.3 µg/L. Calibration functions were established for peak areas of the six peptides analyzed at four mass concentration levels and for three replicates each (Fig. 3). For each concentration level, the average and standard deviation were calculated from the measurement values of the replicates and plotted as the average value ± the standard deviation (with y-axis error bars) in the diagrams. Calibration functions are shown for the first (black lines) and second (gray lines) measurement series. Figure 3 shows that there are significant differences between the days for some of the external calibrations. Therefore, it is highly recommended that fresh external calibrations are performed for each day of measurements to account for the variances, as was done in the work described here. In addition, it should be noted that the response of ANG (4–8) is about one to two orders of magnitude lower than the responses of the other peptide fragments, resulting in a slightly distorted appearance of the slopes due to the utilization of different y-axis scales. Correspondingly, Table 3 summarizes the parameters of the calibration functions, where M-1 stands for the first and M-2 for the second measurement series.

After evaluation of peak areas and heights, the concentrations of the respective peptide impurities were calculated for two ANG I materials with the internal IDs OGO.096b and OGO.096c that are conceivable candidates for peptide purity (certified) reference materials.

For impurities such as ANG I (occurring in an isomeric form), ANG II, ANG III, ANG IV, ANG (1–4), and ANG (4–8) both measurement values for peak heights and areas were used to determine absolute concentration values and standard deviations in OGO.096b and OGO.096c candidate materials. Figure 4 shows the three most intense impurities, ANG II, ANG III, and ANG (2–10), in a measurement file recorded during analysis of the OGO.096c material. The peaks are displayed as overlays of ion current chromatograms of the most intense molecular ion species of the peptides.

The results (*m*/*z*, RT, peak height, and peak area) of the quantitative determination of mass concentrations of the peptide impurities are given as average values obtained from three replicates.

The presence of an impurity (ANG I*) isobaric to ANG I in both candidate materials (OGO.096b and OGO.096c) was confirmed by LC-hrMS. Figure 4 shows the ANG I* impurity at a retention time of 32.7 min. The corresponding high-resolution mass spectra confirm the mass of the ANG I* (not shown). ANG I* is potentially an isomer of ANG I in that one amino acid is exchanged for an isobaric amino acid. Preliminary studies on the purity of amino acids showed that "pure" leucine frequently contains the isobaric isoleucine as a major impurity and vice versa. Therefore, it is likely that the leucine was exchanged for the isobaric impurity isoleucine or vice versa during the synthesis of ANG I, forming the isobaric ANG I* impurity.

Table 4 lists the major impurities detected in both candidate materials for ANG I and compares their values in the first (M-1) and second measurement (M-2) series. The peptide ANG II was the only impurity that could be detected at significant mass concentration levels in both candidate materials and that could be quantified by external calibration. Traces of the peptides ANG III, ANG IV, ANG (1–4), and ANG (4–8) were also observed, but the amounts were too small to be reliably quantified.

An average response factor was established through a "median calibration function" for the estimation of the

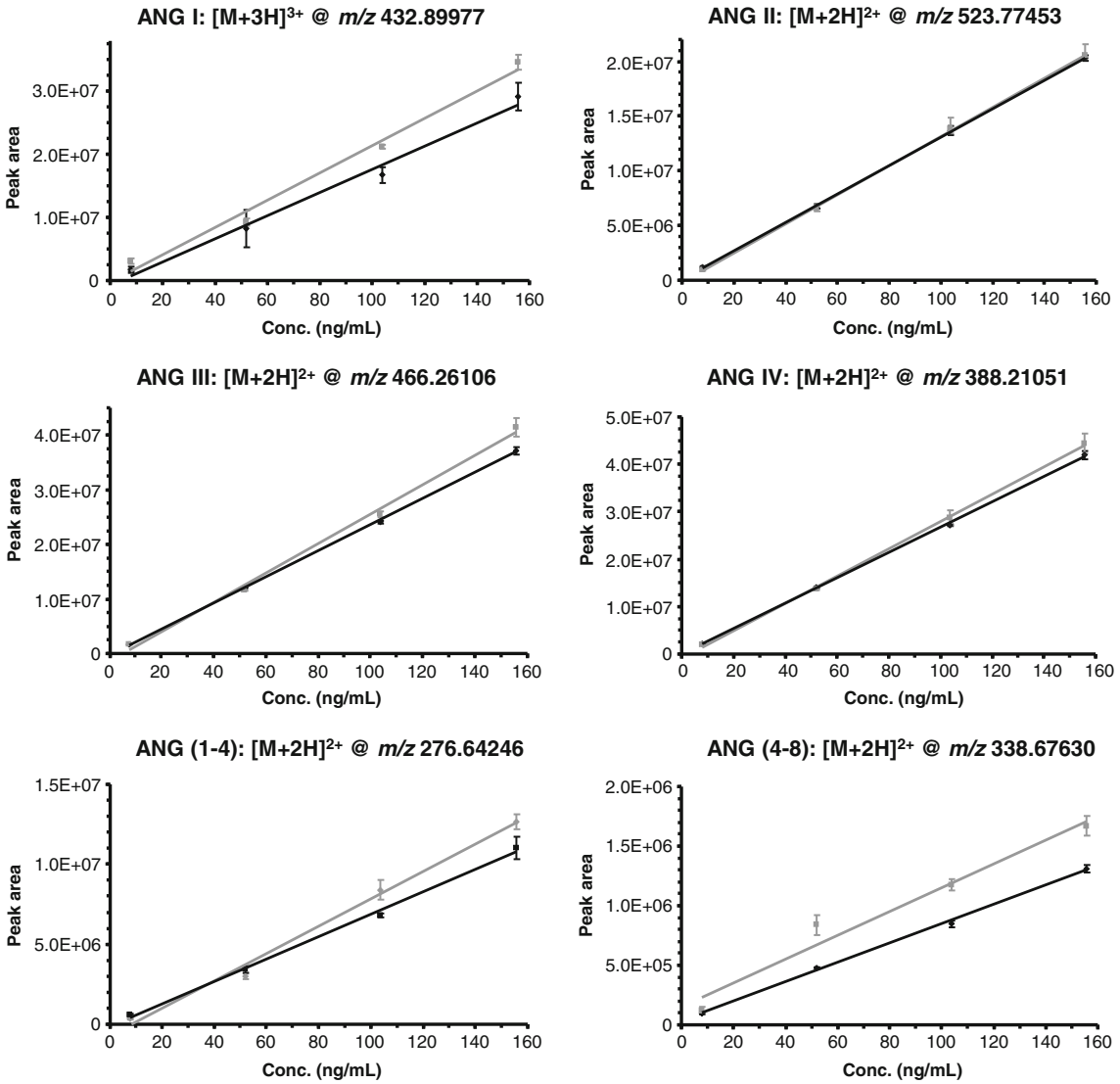

Fig. 3 Calibration curves for selected ANG peptides. For the first (*black lines*) and second (*gray lines*) measurement series, peak areas from the indicated molecular ions of the peptides were used to plot the diagrams. *Error bars* show standard deviations

mass concentrations of ANG I-related peptides for which no original standards were available. The median calibration function was established using the calibration functions obtained from the commercially available original standards for the peptides ANG I, ANG II, ANG III, ANG IV, ANG (1–4), and ANG (4–8), resulting in a slope of 11922 cts nL/ng and an intercept of 54422 cts. However, for most of the detected peptides, mass concentration values that are too low or high may be obtained in calculations using the average response factor. Hence, it can only be used for a rough estimation of the peptide mass concentrations, employing a conservative measurement uncertainty of about 50 %. Two additional related structure impurities, ANG (2–10) and the ANG I isomer, were identified and quantified in both materials using the average response factor (Table 5).

LC-hrMS/MS analysis of other impurities in the ANG I candidate materials

Both ANG I materials OGO.096b and OGO.096c were analyzed with a similar data-dependent LC-hrMS/MS method as that used to characterize the commercially available ANG I standard, as described in "LC-hrMS/MS analysis of the impurities of a commercial ANG I material." In addition to the related structure impurities described above, other possible modifications were investigated, such as oxidation, decarboxylation, phosphorylation, and hydroxylation, depending on which amino acids in the peptide sequence are amenable to such alterations.

A modified analog of ANG I with an *m/z* of 438.23178 for the $[M+3H]^{3+}$ ion was detected in OGO.096c at an RT of

Table 3 Parameters of the calibration functions for six original angiotensin peptides, established in the first (M-1) and second (M-2) measurement series (R^2 being the coefficient of determination)

Peptide	Series	Peak areas			Peak heights		
		Slope (cts s mL ng^{-1})	Intercept (cts s)	R^2	Slope (cts mL ng^{-1})	Intercept (cts)	R^2
ANG I	M-1	183372	−658476	0.9865	9856	−30048	0.9933
	M-2	216377	−217304	0.9865	12587	−33204	0.9825
ANG II	M-1	129793	127761	0.9999	7591	22742	0.9997
	M-2	133420	−193974	0.9995	7937	9204	0.9960
ANG III	M-1	238792	−277511	0.9996	14465	−23048	0.9995
	M-2	269420	−1449380	1.0000	17212	−113679	0.9885
ANG IV	M-1	266748	59075	0.9993	15204	47576	0.9896
	M-2	287910	−796668	0.9994	17303	−58906	0.9983
ANG (1–4)	M-1	70186	−129507	0.9966	4334	−9853	0.9875
	M-2	85565	−717245	0.9917	6344	−49937	0.9929
ANG (4–8)	M-1	8112	36202	0.9981	461	4658	0.9881
	M-2	9970	155528	0.9672	588	10689	0.9692

44.16 min during the column clean-up step. Manual peptide sequencing as depicted in Fig. 5 resulted in a primary sequence of DRVYIHP**Y**HL, indicating that the phenylalanine in ANG I (DRVYIHPFHL) had been hydroxylated to tyrosine. However, the signal intensity of less than 100,000 cps was too low to allow quantification.

Another minor impurity with an *m/z* of 456.90738 for $[M+3H]^{3+}$ was detected in OGO.096c at an RT of 44.64 min during the column clean-up step. The primary sequence PFEANHED could only be partly predicted by manual peptide sequencing. The peptide is clearly part of neither the ANG I nor the precursor angiotensinogen (AGT) comprising 452 amino acids nor its signal peptide comprising 33 amino acids. Database searches using UniProt [20] did not result in any conclusive evidence for the origin of the peptide fragment.

Conclusions

The mass spectrometric properties of a commercial ANG I standard were first studied by direct infusion into a LTQ-Orbitrap XL tandem mass spectrometer.

Besides determining various singly and multiply charged ion species of the intact molecule and its amino acid sequence, several related peptide fragments originating from sequential losses of N- or C-terminal amino acids were also observed. To simulate the degradation and alteration of the ANG I peptide during long-term storage in liquid solvent, aliquots of the standard were stored at 90 °C for up to 143 h and then analyzed by infusion MS/MS. Apart from the main substance ANG I, ten shortened peptides could be detected. During the infusion MS experiments, all of the substances in the mixture entered the ion source of the mass spectrometer at the same

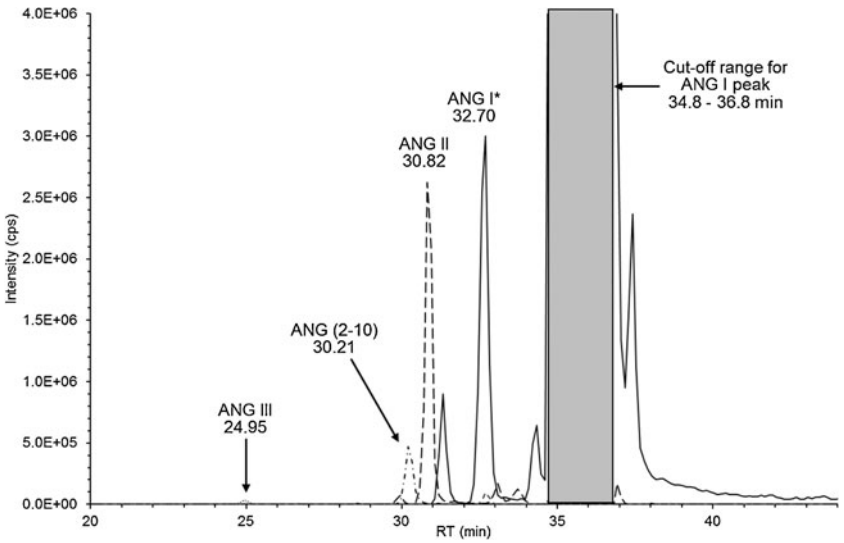

Fig. 4 Overlay of five extracted ion current chromatograms of diagnostic molecular ions of ANG I and an ANG I isomer (*ANG I**) ($[M+3H]^{3+}$ at *m/z* 432.89977, *full line*), ANG II ($[M+2H]^{2+}$ at *m/z* 523.77453, *long dashed line*), ANG III ($[M+2H]^{2+}$ at *m/z* 466.26106, *dotted line*), ANG (2–10) ($[M+2H]^{2+}$ at *m/z* 591.33255, *dashed and dotted line*), and ANG IV ($[M+2H]^{2+}$ at *m/z* 388.21051, *short dashed line*) obtained from replicate measurements of OGO.096c material. Mass tolerance was±5 ppm

Table 4 Quantitative determination of impurities in both ANG I candidate materials (OGO.096b and OGO.096c) on two different days (measurement series M-1 and M-2) by external calibration and evaluation of peak areas

Material (Internal ID)	OGO.096b		OGO.096c	
Series	M-1	M-2	M-1	M-2
Impurity	ANG II	ANG II	ANG II	ANG II
Measured *m/z*	523.77423	523.77511	523.77401	523.77543
Charge state	2	2	2	2
Mean peak area (cts s)	2.50×10^6	2.21×10^6	4.53×10^7	6.26×10^7
Mean impurity mass fraction (mg impurity/ g ANG I)	0.198	0.192	4.41	5.14
SD of impurity mass fraction (mg impurity/ g ANG I)	0.007	0.010	0.08	0.21

time and were ionized together. It was therefore impossible to distinguish between inherent peptides and smaller peptides that were generated by in-source fragmentation of larger peptides.

An LC-hrMS/MS method was therefore developed and optimized for the sensitive and selective detection of ANG I and selected peptides, such as ANG II, ANG III, ANG IV, ANG (1–4), and ANG (4–8). This instrument method also featured an automatic data-dependent recording of MS/MS spectra of possible molecular ions of related peptides which were pre-selected in the same analysis run. The method also allowed for the determination of impurities in high concentrations of ANG I standards without contaminating the mass spectrometer.

The study of artificially altered ANG I demonstrated the possibility that peptide mass fractions or purity values assigned by amino acid analysis could be overestimated unless a correction is made for potential peptide impurities containing the same number of selected amino acids as the model peptide. The analyses of the artificially altered ANG I material showed that primary structures of five other related peptide fragments found by LC-hrMS/MS also contained all four of the amino acids [valine (V), isoleucine (I), proline (P), and phenylalanine (F)] shown in Table 6. These four stable amino acids are frequently selected for amino acid analysis leading to the purity value assignment of the parent peptide or small protein. In addition, nine more ANG I fragments have been observed that contain from one to three of the four amino acids of interest. The applied method can be modified and used for the identification and determination of impurities of other peptides or small proteins.

In addition, the LC-hrMS/MS method was successfully applied to two ANG I candidate reference materials. It was possible to estimate the amount of peptide-related impurities on a quantitative basis using external calibration functions that had been established for six peptides (for which original standards were available). Further peptide fragments could only be quantified on a semi-quantitative basis through estimation of the mass concentrations obtained by applying a "mean calibration function" based on average response factors. The mass concentration values obtained for the impurities in the standards may be considered first estimates only.

Table 5 Quantitative estimation of impurities in both ANG I candidate materials (OGO.096b and OGO.096c) on two different days (measurement series M-1 and M-2) using a mean external calibration function

Material (Internal ID)	OGO.096b		OGO.096c	
Series	M-1	M-2	M-1	M-2
Impurity	ANG (2–10)	ANG (2–10)	ANG (2–10)	ANG (2–10)
Measured *m/z*	394.55665	394.55750	394.55667	394.55787
Charge state	3	3	3	3
Mean peak height (cts)	7.07×10^6	6.07×10^6	4.06×10^6	3.09×10^6
Mean impurity mass fraction (mg impurity/ g ANG I)	6.3	5.1	3.6	2.6
Impurity	ANG I isomer	ANG I isomer	ANG I isomer	ANG I isomer
Measured *m/z*	432.89859	432.89995	432.89915	432.90019
Charge state	3	3	3	3
Mean peak height (cts)	2.21×10^6	1.57×10^6	3.04×10^6	2.50×10^6
Mean impurity mass fraction (mg impurity/ g ANG I)	1.9	1.4	2.7	2.2

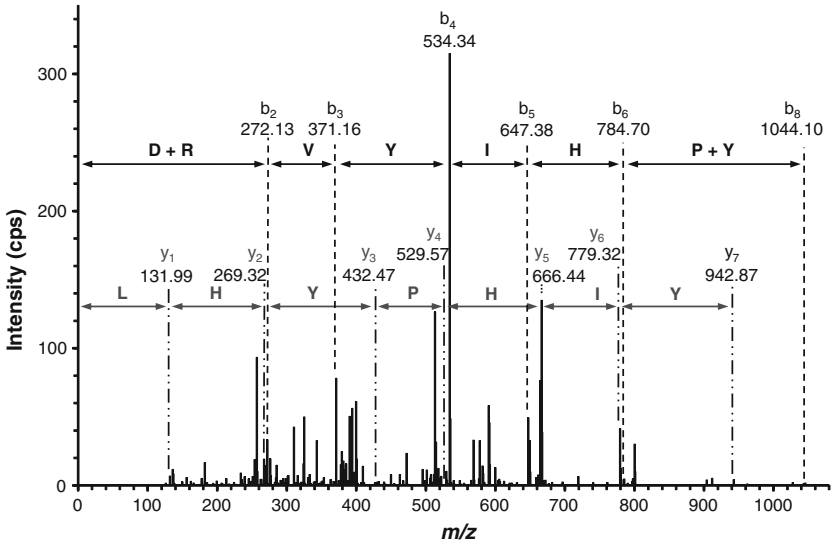

Fig. 5 MS/MS spectrum of the $[M+3H]^{3+}$ ion of a metabolite (DRVYIHP**Y**HL) of ANG I (DRVYIHPFHL) eluting at RT 44.16 min in a data-dependent LC-hrMS/MS experiment

Major related structure impurities of a typical candidate material of ANG I (OGO.096c) have been identified as ANG II, ANG (2–10), and ANG I isomer, with mass fraction impurities of about 4.8±0.7 mg/g (external calibration with an original standard), 3.1±1.0 mg/g, and 2.5±0.5 mg/g (approximation by average response factor), respectively. The expanded uncertainties are the spans of the values obtained for both days, reflecting the intermediate precision contribution at a confidence interval of approximately 95 %. Additional comprehensive screening for modifications (oxidation, etc.) of ANG I using accurate mass measurements only resulted in the identification of a peptide that shows the same primary structure as ANG I but with phenylalanine replaced by tyrosine. However, the response was insufficient for quantification.

Table 6 LC-hrMS/MS results for an altered ANG I material, and the potential impact on a value assignment by amino acid analysis employing isoleucine, phenylalanine, proline, and valine

Peptide	Alias	1 Asp	2 Arg	3 Val	4 Tyr	5 Ile	6 His	7 Pro	8 Phe	9 His	10 Leu	MW (g/mole)	Remark
ANG I	ANG (1-10)	D	R	V	Y	I	H	P	F	H	L	1295.8	√
ANG III	ANG (2-8)		R	V	Y	I	H	P	F			930.5	√
	ANG (2-7)		R	V	Y	I	H	P				783.4	√
ANG IV	ANG (3-8)			V	Y	I	H	P	F			774.4	n.d.
	ANG (2-10)		R	V	Y	I	H	P	F	H	L	1180.7	√
	ANG (3-10)			V	Y	I	H	P	F	H	L	1024.6	√
	ANG (4-10)				Y	I	H	P	F	H	L	925.5	√
	ANG (5-10)					I	H	P	F	H	L	762.4	√
	ANG (6-10)						H	P	F	H	L	649.3	√
	ANG (7-10)							P	F	H	L	512.3	√
	ANG (8-10)								F	H	L	415.2	√
	ANG (9-10)									H	L	268.2	√
	ANG (1-9)	D	R	V	Y	I	H	P	F	H		1182.6	√
ANG II	ANG (1-8)	D	R	V	Y	I	H	P	F			1045.5	√
	ANG (1-7)	D	R	V	Y	I	H	P				898.5	n.d.
	ANG (1-6)	D	R	V	Y	I	H					801.4	√
ANG V	ANG (1-5)	D	R	V	Y	I						664.4	n.d.
	ANG (1-4)	D	R	V	Y							551.3	n.d.
	ANG (1-3)	D	R	V								388.2	√
	ANG (1-2)	D	R									289.1	n.d.

n.d. not detected

The sum of the impurity mass fractions was about 10.4± 1.3 mg/g. If the value assignment of a solution of this ANG I candidate material is performed by amino acid analysis using the abovementioned amino acids without taking the purity into account, a bias of about 1 % would be introduced. The current study only shows the preliminary results. The outcome using artificially altered ANG I indicates that a much higher quantity of related structure impurities containing certain amino acids can be expected. More accurate mass fraction quantification of peptide-related impurities in candidate ANG I standards, as identified by LTQ-Orbitrap XL, are currently ongoing.

The preliminary studies clearly demonstrate the importance of purity determinations of intact peptides in establishing reference measurement systems traceable to the SI. Approaches for the determination of peptide purity are currently being studied by the BIPM and will be further investigated by several National Metrology Institutes within the framework of the Bioanalytical Working Group (BAWG) of the Consultative Committee for Amount of Substance (CCQM).

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