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Quantification of ACE inhibiting peptides in human plasma using high performance liquid chromatography–mass spectrometry

Chris J. van Platerink [a,b,*], Hans-Gerd M. Janssen [a,c], Roos Horsten [a], Johan Haverkamp [a,b]

[a] *Advanced Measurement and Imaging, Unilever Research Laboratory, PO Box 114, Vlaardingen 3130AC, The Netherlands*
[b] *Biomolecular Mass Spectrometry Group, Bijvoet Centre for Biomolecular Research, Faculty of Chemistry, University of Utrecht, Sorbonnelaan 16, 3584 CA, Utrecht, The Netherlands*
[c] *Polymer-Analysis Group, van't Hoff Institute for Molecular Sciences, University of Amsterdam, Nieuwe Achtergracht 166, 1018 WV Amsterdam, The Netherlands*

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Abstract

An HPLC-MRM–MS method was developed for the quantification of 17 small ACE inhibiting (ACEI) peptides in plasma samples collected from human volunteers after the consumption of a peptide-enriched drink. The assay shows the high selectivity and sensitivity necessary to monitor small changes in the levels of the ACEI peptides after consumption of drinks developed to effect lowering of the blood pressure. Four different sample preparation methods were tested and evaluated. The final sample preparation method selected is simple and effective and consists mainly of the removal of proteins by acidification and heating, followed by a large volume injection. Additional sample preparation steps such as solid phase extraction and liquid/liquid partitioning were studied. Although they resulted in cleaner extracts, losses of specific peptides such as SAP were frequently seen. The isotope labeled form of one of the peptides to be quantified, [U^{13}C]IPP, was used as an internal standard. The limit of detection of the assay is below 0.01 ng ml^{-1}. The limit of quantification is between 0.05 and 0.2 ng ml^{-1}, which is approximately 10% of the expected peptide concentration in plasma based on a normal diet. The intra- and inter-day relative standard deviations for all peptides have shown to be below 25% and the method has an accuracy of better than 75%. The long-term stability is good. At least 200 samples could be analysed before the system had to be cleaned. The assay has been successfully applied to blood samples collected from volunteers during a human trial.

Keywords: Bioactive peptides; LC–MS; Plasma; Angiotensin-I-converting enzyme inhibitors

1. Introduction

One of the key enzymes in the regulation of blood pressure is the angiotensin-I-converting enzyme (ACE). The ACE enzyme converts angiotensin-I into the potent vasoconstrictor angiotensin-II. Many protein-containing food products, ranging from fish and meat to milk and maize, are known to yield ACE-inhibitory peptides upon in-vivo-proteolysis. So far, a wide range of different peptides with ACE-inhibitory effects has been identified using peptide purification methods and in vitro ACE inhibition assays [1–5], or more sophisticated on-line techniques [6]. Some of the identified peptides have been synthesised and proven to be active in both in vitro ACE-inhibition assays as well as in relevant in vivo animal models for hypertension.

Although many peptides from food proteins have been shown to inhibit ACE in vitro and also lowered blood pressure in in vivo studies, the proposed properties of these peptides in real life situations still need to be demonstrated. To exert their physiological effect in the body, the peptides need to be absorbed from the intestine and enter the blood circulation in an active form. So far, there is no direct evidence that any of the identified peptides are indeed absorbed and reach the blood circulation. Therefore, it is extremely important to demonstrate the presence of ACE-inhibiting peptides in blood after the consumption of a food product containing these peptides. In order to be able to measure these peptides in plasma, highly sensitive and accurate methods are required. The expected concentration levels are in the picogram per millilitre range.

* Corresponding author. Tel.: +31 10 4605478; fax: +31 46 05310.
E-mail address: chris-van.platerink@unilever.com (C.J. van Platerink).

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Peptide identification, and to a lesser extent quantitation, has received a great deal of attention in recent years due to the rapidly expanding interest in proteomics [7,8]. For the current work, however, that research is unfortunately only of limited- or indirect relevance. This is because of the different matrices involved and the nature of the underlying question, i.e. identification of proteins versus the quantification of small peptides with known structures. A second area where peptide analysis is performed is in metabonomics research where peptide profiles of patients suffering from a specific disease are compared to that of healthy patients [9,10]. The peptides analysed are relatively large with a sequence of at least eight residues and molecular ions of 0.8–5 kDa. Finally, an area where the quantification of known peptides in biological fluids is relevant is in the study of peptidic drugs. For example Yang et al. [11] developed a method for the quantitative analysis of an opiod pentapeptide and its cyclic prodrug in rat plasma. Chavez-Eng et al. [12] measured a cyclic hexapeptide with known antifungal properties in human plasma. In these studies the plasma samples are separated using HPLC with UV, electrochemical, fluorescence or mass spectrometric detection. Nowadays the very sensitive mass spectrometric detector is preferred over fluorescence due to its much higher selectivity and the possibility to work without derivatisation. The sample preparation methods applied generally consist of removing proteins by precipitation or other techniques, followed by isolation and concentration of the peptide fraction. The typical detection limits of such procedures are in the low ng ml^{-1} range.

The limitations of the methods mentioned above are the relatively large volumes of blood that are needed and/or the extensive cleanup procedures that must be applied to isolate and concentrate the peptides of interest. Moreover, the target peptides in these peptidic drugs studies are relatively large, which is advantageous for the selection of ions for tandem-MS operation. Finally, measurements are also simpler if unique peptides, i.e. peptides with sequences that do not occur in the proteins normally consumed, have to be measured.

In our study healthy volunteers consumed fermented milk enriched in ACEI peptides in a human trial performed over a time period of several weeks. During the study the blood pressure was measured and blood samples were collected for quantification of previously identified ACEI peptides. Considering the peptide intake and assuming 0.01–1% effective absorption and transport to the systemic blood circulation, the expected concentration of the peptides in the blood should be in the high pg ml^{-1} range. In order to be able to quantify peptides at this very low level, the desired detection limit was set at 0.01 ng ml^{-1}.

The subject of this paper is the development and validation of an analytical method for the quantification of small ACEI peptides in plasma in the pg ml^{-1} range. Liquid chromatography with atmospheric pressure ionisation tandem mass spectrometry is selected as the quantification method. Various methods for sample clean-up are evaluated with emphasis on suitability for above mentioned sensitivity range and long term stability. The full set of ACEI active peptides consists of 17 peptides with sequence lengths between 2 and 5 residues and different hydrophobicity. Statistical information on the performance of the HPLC–MS–MS method is reported and its inherent limitations are discussed. Finally the application of the method to the analysis of authentic blood samples is demonstrated.

2. Experimental

2.1. Materials and methods

2.1.1. Standards and chemicals

The peptides IPP and VPP (purity >98%) were purchased from Bachum (Budendorf, Switzerland). The internal standard used was [U^{13}C]IPP (fully ^{13}C labelled in I only) and was purchased from Bio Peptide (San Diego, USA). All other peptides were synthesised at the University of Utrecht (the Netherlands). The sequences of the peptides studied are given in Table 1. The purity of each peptide was determined in-house using HPLC-UV–MS. All purities were better than 90%. Acetonitrile, trifluoro acetic acid, propionic acid and 2-propanol were purchased from Merck (Amsterdam, the Netherlands). Porcine and bovine plasma were obtained from a local slaughter house.

2.1.2. Solid phase extraction

Octadecyl modified silica solid phase extraction cartridges (3 ml, 500 mg) were purchased from Waters (Etten-Leur, the Netherlands). The cartridges were preconditioned with 4 ml of methanol followed by 4 ml of Milli-Q water. After loading of the sample, the cartridge was washed with 1 ml of Milli-Q water and the peptides were eluted using 4 ml of methanol. The collected methanol fraction was evaporated to dryness under a stream of nitrogen at 40 °C and reconstituted in 500 μl of Milli-Q water.

2.1.3. Standard preparation

Peptide standard solutions were prepared either in Milli-Q water or in a fivefold diluted SPE extract of bovine plasma (vide infra). The bovine plasma extract was prepared by addition of 20 μl of a 10% aqueous TFA solution to 1 ml of plasma. The mixture was vortexed for 2 min followed by heating at 95 °C for 2 min. After cooling and centrifugation at 13,000 rpm the supernatant phase was purified by passing it through a C18 Sep-Pak SPE column (Waters Etten-Leur, the Netherlands). One millilitre of plasma was applied onto a 20 ml/5 g cartridge previously conditioned with 20 ml of methanol and 20 ml of Milli-Q water. The protein pellet in the centrifugation tube was discarded. Methanol (4 ml) was applied for elution of the cartridge. The methanol fraction was collected and evaporated under a stream of nitrogen at room temperature. The residue was redissolved in 5 ml of Milli-Q water.

Calibration standards were prepared starting from a mixture of the standard peptides with a concentration of 500 μg ml^{-1} for each peptide in Milli-Q water. Subsequent dilutions were made in the bovine plasma extract to yield the following concentrations: 0.01, 0.05, 0.1, 0.2, 0.5, 1 and 2 ng ml^{-1}. Each solution contained the internal standard [U^{13}C]IPP at a concentration of 1 ng ml^{-1}.

Table 1
Sequences, MRM conditions and product ion ratios of the peptides measured in the assay

Peptide	Precursor ion *m/z*	*V*[a]	Product ion 1 *m/z*	CeV-1[b]	Product ion 2 *m/z*	CeV-2[c]	Ratio ion 1/ion 2
VPP	312.2	25	213.1	18	169.1	18	1.6/1
IPP	326.2	25	213.1	18	183.1	18	5.5/1
LPP	326.2	25	213.1	18	183.1	18	3.4/1
IPPL	439.2	25	326.2	16	183.1	16	2.5/1
HLP	366.2	25	251.2	14	86.0	30	15.3/1
HLPLP	576.3	60	251.2	24	183.1	24	4.7/1
VAP	286.2	25	116.2	12	70.0	15	13.2/1
SAP	274.1	25	187.1	11	70.0	17	11.6/1
IIAEK	573.3	60	347.2	20	86.0	30	12.7/1
FY	329.1	30	120.0	18	182.1	14	5.5/1
VF	265.1	30	72.0	16	166.1	11	1.3/1
VY	281.1	26	182.1	11	72.0	17	1.2/1
IY	295.1	23	86.0	18	182.1	11	3.4/1
AW	276.1	22	188.1	20	205.1	20	10.1/1
IW	318.2	24	205.1	14	86.0	18	1.8/1
KVLPVP	652.4	40	197.1	20	537.4	13	4.2/1
LW	318.2	24	205.1	14	86.0	18	1.2/1
[U^{13}C]IPP	332.2	25	213.1	18	189.1	18	2.2/1

[a] Cone voltage.
[b] Collision energy product ion 1.
[c] Collision energy product ion 2.

2.1.4. Instrumentation

All analyses were performed on a Waters 2790 HPLC coupled to a Micromass Quattro-Premier triple quadrupole mass spectrometer (Waters, Almere, the Netherlands).

2.1.5. Chromatographic conditions

All analyses were performed on a 150 mm × 2.1 mm Inertsil 5 ODS3 column with a particle size of 5 μm (Varian, Bergen op Zoom, the Netherlands), equipped with a 10 mm × 4.6 mm a reversed phase C8 guard column (Waters, Etten-Leur, the Netherlands). Mobile phase A consisted of 0.1% trifluoro acetic acid (TFA) in Milli-Q water. Mobile phase B consisted of 0.1% TFA in acetonitrile. The initial eluent composition was 100% A. The eluent was kept at 100% A for 5 min and increased to 5% B in 10 min, followed by a linear gradient to 30% B in 15 min. Then a linear gradient was used to 70% B in 5 min, and the eluent composition was kept at 70% B for another 5 min. The eluent was reduced to 100% A in 1 min and the system was allowed to equilibrate for 9 min. The total run time was 50 min. The eluent flow was 0.2 ml min^{-1} and the column temperature was set at 60 °C. The UV trace was recorded at 215 nm. The eluent of the first 5 min was directed to waste to avoid contamination of the mass spectrometer with salts and other highly polar compounds. The injection volume ranged from 10 to 150 μl depending on the purity of the sample after the sample preparation. If after sample pre-treatment the solution was viscous or unclear, a volume of 10 μl was injected. In all other cases the injection volume was 150 μl.

2.1.6. Post column additive

To overcome suppression of the ionisation due to the presence of TFA in the eluent, a mixture of propionic acid and 2-propanol (70/30, v/v) was added post column using a separate pump. The solvent delivery system used was a P1000 isocratic pump (Spectra-Physics, Darmstadt, Germany). The flow rate was 0.05 ml min^{-1}. A piece of 30 cm of fused silica tubing (75 μm i.d.) was used as a restrictor between the pump and the post-column T-piece in order to maintain a stable flow rate.

2.1.7. Mass spectrometric conditions

All measurements were carried out using high performance liquid chromatography-atmospheric pressure ionisation-multiple reaction monitoring mass spectrometry (HPLC-API-MRM–MS) in positive ionisation mode. The capillary voltage was set at 4 kV. The source temperature was kept at 100 °C and the nebulizer temperature at 250 °C in order to prevent in-source condensation, especially at the starting eluent composition of 100% water. The desolvation and cone gas flows were 355 and 188 l h^{-1}, respectively. Argon was used as collision gas at a gas pressure of 2.3e−3 mbar. The dwell time was set at 0.5 s for each peptide, with an interscan time of 0.05 s. The optimum cone voltages and collision energies were determined for each peptide individually prior to the start of the experiments. Peptides were identified based on their retention time, their molecular ion and the ratio of two different characteristic product ions for each peptide relative to those of the model compounds. A maximum variation of 0.2 min for the elution time and 10% for the product-ion ratios was accepted. Detailed information on the product-ions, cone voltages and collision energies is given in Table 1.

3. Results and discussion

With the highly selective and sensitive state of the art LC–MS-MS systems plasma samples can generally be analysed after only limited sample pretreatment. In many cases protein removal by solvent or heat precipitation suffices [15]. In a first series of experiments we studied whether this simple “dilute and

Table 2
Recovery of IPP from spiked porcine plasma after solvent precipitation

Plasma/solvent ratio	Recovery (%)
Plasma/methanol 1:2	105
Plasma/methanol 1:1	93
Plasma/acetonitrile 1:2	1
Plasma/acetonitrile 1:1	26
Plasma/ethanol 1:2	73
Plasma/ethanol 1:1	73

Table 3
Recovery of the peptides VF, SAP, IPP, IIAEK and HLPLP using SPE purification

Concentration (ng ml^{-1})	Recovery (%)				
	VF	SAP	IPP	IIAEK	HLPLP
10	96	35	96	103	73
50	100	54	76	95	75
100	97	33	85	71	77
500	80	35	72	70	70
1000	87	51	69	86	73

shoot" method [15] provided sufficient sensitivity for the current analysis. To minimize the usage of human plasma required for the method development porcine plasma, known to be a reasonable alternative for human plasma, was used in this part of the work.

The initial sample preparation consisted of protein precipitation using organic solvents. Three different solvents were tested: methanol, ethanol and acetonitrile with plasma/solvent ratio's of 1:1 or 2:1 followed by centrifugation at 13,000 rpm for 30 min. The volume of plasma pretreated was 1 ml. The supernatant was concentrated five times under a nitrogen gas flow at ambient temperature. The recovery was determined using porcine plasma spiked at a high level (60 μg ml^{-1}) with IPP. An injection volume of 10 μl was used. The results are given in Table 2. The data show that the best recovery (∼100%) was achieved with methanol. Subsequent analyses were performed using plasma/methanol in a ratio of 1:2. The limit of detection was determined using porcine plasma spiked with IPP and KVLPVP in the concentration range of 0.04–4 ng ml^{-1} and using an injection volume of 150 μl. The LODs for KVLPVP and IPP were 0.4 and 4 ng ml^{-1}, respectively. This was not low enough to quantify the peptides at the desired concentration level. Another drawback of this sample preparation protocol was that contamination of the MS source already occurred after approximately 10 injections causing a strong decrease in sensitivity. In order to improve the efficiency of the protein removal step, a solid phase clean up step was incorporated in the method. After centrifugation at 13,000 rpm for 30 min, 1 ml of the supernatant of the spiked porcine plasma samples was applied to a pre-conditioned SPE cartridge. The SPE eluent was evaporated under nitrogen at 40 °C and the residue was then reconstituted in 500 μl of Milli-Q water. After reconstitution the solution was cloudy, most likely as a result of proteins and lipids not being removed during the precipitation and SPE steps. Therefore, it was decided to use a more rigorous method to remove the proteins from the plasma. In this method protein precipitation was obtained by acidification of the plasma and temperature treatment. Twenty microlitres of a 10% aqueous TFA solution was added to 1 ml of plasma. This mixture was then heated at 100 °C for 2 min. After cooling to ambient temperature the SPE step described above was applied. After evaporation of the solvent and reconstitution in 500 μl of Milli-Q water the solution was clear, indicating that full removal of proteins and fat was obtained.

The recovery of the method outlined above was determined by spiking human pool plasma with the peptides VF, SAP, IPP, IIAEK and HLPLP at concentrations of 10, 50, 100, 500 and 1000 ng ml^{-1} plasma. The results of the experiments are summarised in Table 3. The results show that the acid/heat/SPE purification method can be used for hydrophobic peptides. Hydrophilic peptides such as, e.g. SAP, unfortunately, have poor recoveries because they are not, or only partly retained on the SPE column. Experiments with model compounds in pure water confirmed this statement. The long term reproducibility of the method appeared to be poor, with standard deviations of approximately 50%. To overcome this problem, the SPE step was omitted from the method. The final, strongly simplified method consisted of the addition of 20 μl of a 10% TFA solution to 1 ml of plasma. After vortexing for 30 s the mixture was heated to 100 °C for 2 min, rapidly cooled down using running cold water and centrifuged for 30 min at 13,000 rpm. One hundred and fifty microlitres of the approximately 200 μl of supernatant was then injected onto the HPLC-column. Such large injection volume did not result in significant peak broadening when compared to injections of smaller sample volumes.

During the experiments it appeared that the peptide levels decreased during storage in aqueous solutions, especially at concentration levels below 10 ng ml^{-1}. This even occurred if samples were stored at −20 °C and is probably due to binding of the peptides to active sites of the glass vial. Different vial types were tested, including polyethylene and silylated glass vials, however, without significant improvement. The best results were obtained when the peptides were dissolved in five times diluted SPE-purified bovine plasma. Most likely, the excess peptides present in the background efficiently prevent adsorption of the peptides of interest on the active sites.

The final method was validated according to the procedure described by Shah et al. [13]. Calibration data were generated using either the SPE extract of bovine plasma or human pool plasma spiked with 17 peptides that can be formed from bovine milk proteins upon digestion, at concentrations of 0, 0.01, 0.05, 0.1, 0.2, 0.5, 1 and 2 ng ml^{-1}. The concentration of the [U^{13}C]IPP internal standard in all samples was 1 ng ml^{-1}. The protein sources and the in-house determined Matsui IC50 ACEI values [14] of the peptides are given in Table 4. The calibration curves were constructed from the peak area ratios of the analyte relative to the internal standard versus concentration. The concentrations of the peptides in the spiked pool plasma sample were calculated using a linear equation fitted through the different calibration curves. The limit of detection was defined as the lowest detectable chromatographic peak with a signal-to-

Table 4
Protein sources and Matsui activities of spiked peptides

Peptide	Protein source	Position	Matsui IC50 value (μM)
VPP	β-Casein	84–86	2.5
IPP	β-Casein	74–76	2
LPP	β-Casein	151–153	10
IPPL	β-Casein	74–77	>50
HLP	β-Casein	134–136	>100
HLPLP	β-Casein	134–138	17
VAP	α-s1-Casein	25–27	1
	Lactotransferrin	610–612	
FY	α-s1-Casein	145–146 and 153–154	10
	α-s2-Casein	103–104	
VF	α-Lactalbumin	27–28	5–10
	α-s1-Casein	31–32	
	β-Lactoglobulin	81–82	
	Lactotransferrin	83–84 and 233–234	
VY	α-s2-Casein	198–199	5
	β-Casein	59–60	
	β-Lactoglobulin	41–42	
IY	Lactotransferrin	100–101 and 418–419	0.5
AW	α-s1-Casein	163–164	5
IW	α-Lactalbumin	78–79	0.5
	Lactotransferrin	286–287	
LW	α-s1-Casein	198–199	5

noise ratio of 3:1. The limit of quantification was defined as the lowest concentration that could be measured with a maximum day-to-day relative standard deviation of 25% and a maximum deviation from the nominal value of 25%. The LOD and LOQ were determined by analysing the spiked plasma samples on five different days covering a time period of two weeks. The intra- and inter-day repeatability and reproducibility and the accuracy of the method were determined by analysing the spiked extract of SPE purified bovine plasma in the concentration range of 0.01–2 ng ml^{-1} five times on five different days covering a time period of 11 days. The accuracy was expressed as the percentage of the measured concentration versus the spiked concentration. The stability of the samples was assessed by analysing 21 spiked plasma samples with concentration levels between 0.01 and 2 ng ml^{-1} stored for 24 or 72 h at 5 and −20 °C. The percentage carry-over was determined by the injection of blank Milli-Q water following the analysis of plasma samples spiked at concentration levels of 0.5 and 5 ng ml^{-1}.

3.1. Linearity

The calibration curves were linear in the concentration range from 0.01 to 2 ng ml^{-1}. The linearity (R^2) for all peptides was better than 0.997. The LOD was below 0.01 ng ml^{-1} for all peptides. The LOQ values ranged between 0.01 and 0.03 ng ml^{-1}. The LOD and LOQ met the target values as described in the introduction. A summary of linearity data of the assay validation is given in Table 5. Representative chromatograms of seven of the peptides studied are given in Fig. 1.

Table 5
Linearity data of peptides detection in human plasma using LC-MRM–MS

Peptide	Limit of detection LOD (ng ml^{-1})	Limit of quantification (LOQ (ng ml^{-1})	Linear regression coefficient (R^2)
VPP	0.01	0.05	0.9987
IPP	0.005	0.01	0.9993
LPP	0.002	0.01	0.9991
IPPL	0.002	0.01	0.9989
HLP	0.001	0.01	0.9994
HLPLP	0.01	0.05	0.9974
VAP	0.01	0.05	0.9978
FY	0.005	0.01	0.9987
VF	0.001	0.01	0.9994
VY	0.001	0.01	0.9990
IY	0.003	0.01	0.9996
AW	0.003	0.01	0.9990
IW	0.005	0.01	0.9988
LW	0.002	0.01	0.9992

3.2. Repeatability, reproducibility and accuracy

Statistical evaluation of the results indicated an acceptable accuracy, repeatability and reproducibility of the method. The repeatability and reproducibility improved significantly at higher concentrations. The intra- and inter-day results were very similar. The results of the inter-day measurements are given in Table 6. For some of the peptides the inter- and intra-day precision values for the LOQ are above the generally accepted limit for quantitative bioanalyses. Despite that the accuracy and precision of the method enable its application for monitoring changes

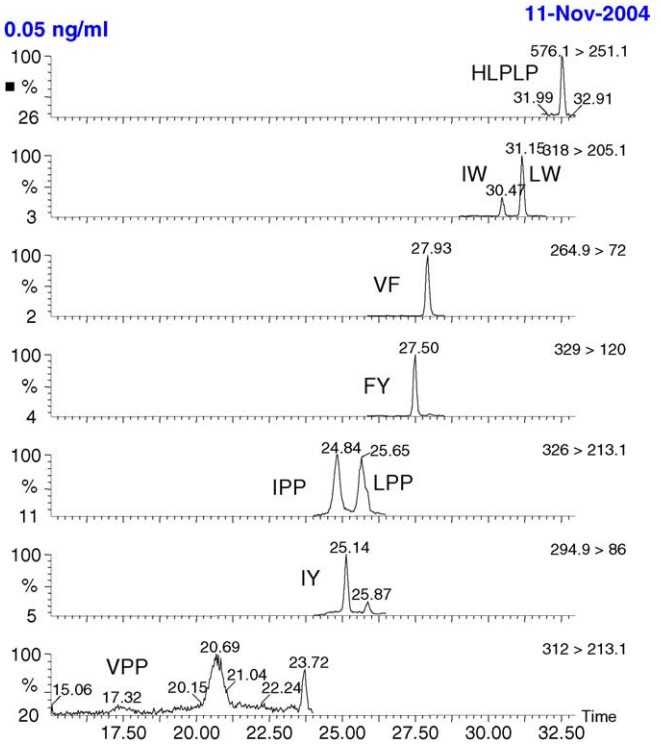

Fig. 1. Typical chromatogram of a standard solution with a concentration of 0.05 ng ml^{-1} for each peptide. For display purposes only the chromatograms of seven randomly selected peptides are given, the chromatograms of the other peptides are very similar.

Table 6
Inter-day precision and accuracy of the assay

Concentration (ng ml^{-1})	VPP		IPP		LPP		IPPL	
	Precision	Accuracy	Precision	Accuracy	Precision	Accuracy	Precision	Accuracy
0.01	29.8	112.1	21.6	99.8	22.1	88.8	22.1	108.8
0.05	23.8	84.8	15.8	101.6	16.7	103.8	16.7	113.8
0.1	13.0	109.6	17.9	103.2	9.5	108.8	9.5	108.8
0.2	4.5	94.0	9.9	99.2	3.4	100.2	3.4	100.2
0.5	2.9	97.0	7.0	95.0	2.2	94.8	2.2	94.8
1	1.9	102.6	4.4	99.8	5.0	96.0	5.0	96.0
2	0.4	99.8	1.5	100.4	0.9	101.4	0.9	101.4

Concentration (ng ml^{-1})	HLP		HLPLP		VAP		FY	
	Precision	Accuracy	Precision	Accuracy	Precision	Accuracy	Precision	Accuracy
0.01	23.0	85.8	26.7	85.8	28.5	119.4	23.6	112.0
0.05	19.9	84.6	19.7	87.2	24.5	101.4	14.7	85.4
0.1	13.8	101.6	21.2	110.8	11.3	118.8	8.3	113.6
0.2	11.3	98.0	4.2	100.6	6.4	95.2	1.4	100.0
0.5	3.5	96.4	6.3	95.2	8.6	94.6	2.0	97.2
1	3.1	104.6	5.1	104.8	5.6	99.8	2.5	104.8
2	0.8	99.2	1.3	98.8	1.5	100.2	0.9	99.4

Concentration (ng ml^{-1})	VF		VY		IY		AW	
	Precision	Accuracy	Precision	Accuracy	Precision	Accuracy	Precision	Accuracy
0.01	21.5	112.1.	23.9	88.8	23.6	84.4	21.1	87.2
0.05	18.8	101.2	21.1	82.2	21.5	89.8	20.5	89.0
0.1	9.3	118.0	9.7	107.8	12.7	108.6	5.0	109.2
0.2	4.9	98.0	3.3	99.2	7.5	95.4	5.9	96.2
0.5	5.0	100.0	4.9	98.6	4.1	98.8	4.3	97.4
1	2.4	105.2	1.7	103.8	4.6	105.6	2.7	103.8
2	0.8	98.8	0.9	99.4	1.7	98.6	0.9	99.4

Concentration (ng ml^{-1})	IW		LW	
	Precision	Accuracy	Precision	Accuracy
0.01	24.8	89.8	22.7	111.7
0.05	17.9	88.0	15.8	108.2
0.1	4.0	112.6	6.2	99.1
0.2	5.5	96.6	5.1	98.6
0.5	3.5	98.8	3.8	99.4
1	2.3	104.4	2.0	99.0
2	1.1	99.2	0.9	98.8

The precision is expressed as the coefficient of variation (C.V., %); $n = 5$.
The accuracy is expressed as [(mean calculated concentration)/(spiked concentration)] × 100.

in the concentration of the peptides upon the intake of a drink enriched in ACE-inhibiting peptides.

3.3. Stability

No significant decrease could be detected in the concentration of peptide solutions prepared in the SPE extract of bovine in concentrations of 0.05 and at 2 ng ml^{-1} stored for 24 and for 72 h at 5 °C or at −20 °C. The variations seen were within the reproducibility limits of the method.

3.4. Carry-over

The percentage carry-over was below 1% for VPP, IPP, LPP, IPPL, VAP, VY, AW, IW and LW measured at 0.5 ng ml^{-1} and at 5 ng ml^{-1}. For FY the carry-over was measured to be 5% at 0.5 ng ml^{-1} and 1% at 5 ng ml^{-1}. The carry-over for HLPLP, VF, and IY was 10% and 1% at concentration levels of 0.5 and 5 ng ml^{-1}, respectively. The relatively high carry-over for the latter peptides was probably caused by partial release of these peptides adsorbed to the column material during preceding runs. This adversely affects the accuracy of the method for these peptides.

3.5. Robustness

The method was used in a human trial where the influence of food peptides in relation to blood pressure was studied. The method proved to be robust. At least 200 plasma samples could be analysed before the MS source had to be cleaned. The

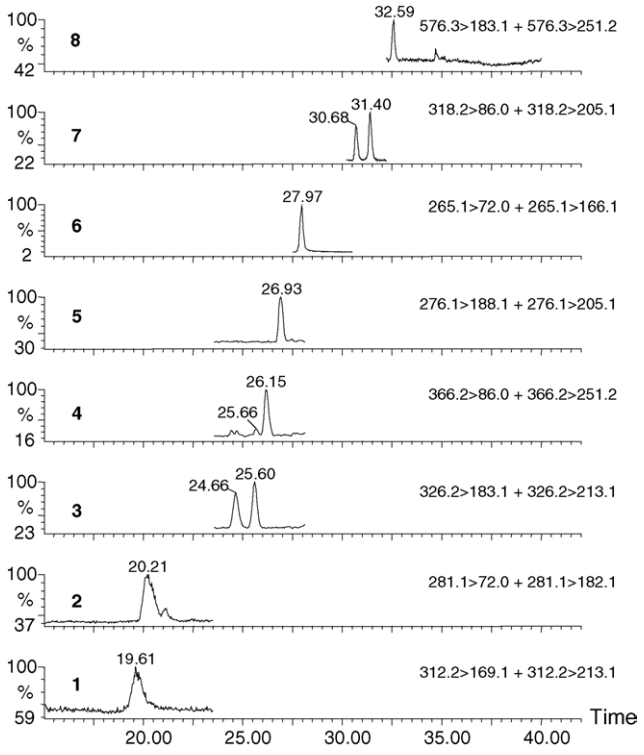

Fig. 2. Typical chromatogram of a plasma sample collected during a human trial. The traces represent the summarized traces of the two product ions monitored for each peptide indicated in the chromatograms. Only the traces of the peptides detected in the sample are displayed. Trace 1: VPP, 2: VY, 3: IPP Rt 24.66 and LPP Rt 25.60, 4: HLP, 5: AW, 6: VF, 7: IW Rt 30.68 and LW Rt 31.40, 8: HLPLP.

HPLC-column did not show any sign of deterioration from this large number of samples. The only precaution that was taken was the replacement of the guard column after 250 samples. The method was applied to more than 1000 plasma samples collected during this human trial. Typical ion chromatograms obtained from a plasma sample collected during the trial are given in Fig. 2. The levels found in this particular sample ranged from 0.05 ng ml^{-1} for VPP to 0.3 ng ml^{-1} for VF. Good peak shapes are seen for all peptides. The slight broadening seen for the early eluting peptides VY and VPP is the result of the large injection volume. The high selectivity of the chromatographic method is nicely reflected in the separation of the peak pairs IPP/LPP and IW/LW. Baseline separation is obtained for both peptide pairs.

The good sensitivity, the high selectivity and the excellent long term stability of the method clearly indicate that the process used for sample preparation, chromatographic separation and detection is in proper balance. This makes the developed method a useful tool in research aimed at the development of novel blood pressure lowering functional foods.

4. Conclusions

An HPLC-MRM–MS method has been developed and validated for measuring 17 ACE inhibiting small peptides in human blood plasma in one single analysis. The sample preparation method was optimised. Various methods for protein removal were compared. The best results were obtained using acidification and heating. The application of SPE for protein removal resulted in poor reproducibilities. The limits of detection and quantification were between 0.001 and 0.05 ng ml^{-1} and are low enough to monitor the peptides at the concentration levels occurring in case of a normal bovine diet. The accuracy is better than 75% and intra- and inter-day relative standard deviations are below 25%, enabling monitoring of even small changes in the concentration levels after consumption of a peptide-enriched drink. The method was successfully applied to large numbers of plasma samples collected during a human trial. At least 200 samples could be analysed before the MS source had to be cleaned making the method practically suitable for application in human trials where large numbers of samples have to be measured in time periods of weeks or months.

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