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Validation of a quantitative assay for human neutrophil peptide-1, -2, and -3 in human plasma and serum by liquid chromatography coupled to tandem mass spectrometry

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ABSTRACT

A quantitative assay for simultaneous measurement of individual human neutrophil peptide-1, -2 and -3 concentrations will aid in exploring the potential of these antimicrobial peptides as biomarkers for various diseases. Therefore, a liquid chromatography–tandem mass spectrometry method has been developed and validated to allow separate quantification of the three human neutrophil peptides in human plasma and serum. Plasma and serum samples (100 μl) were deproteinized by precipitation, followed by chromatographic separation on a Symmetry 300 C_{18} column (50 mm × 2.1 mm I.D., particle size 3.5 μm), using a water–methanol gradient containing 0.25% (v/v) formic acid and human alpha-defensin 5 as internal standard. Tandem mass spectrometric detection was performed on a triple quadrupole mass spectrometer equipped with electrospray ionization. Despite low fragmentation efficiency of the antimicrobial peptides, multiple reaction monitoring was used for detection, though selecting the quaternary charged ions as both precursor and product. The method was linear for concentrations between 5 and 1000 ng/ml with a limit of detection around 3 ng/ml for all peptides. Intra- and inter-assay precisions were 14.8 and 19.1%, respectively, at the lowest measured endogenous concentration (6.4 ng/ml of HNP-1 in plasma), representing the lower limit of quantification of the assay. Recoveries of HNP-1, -2 and -3 from plasma and serum ranged between 85 and 128%. Analysis of serum samples from intensive care patients showed average concentrations of 362, 570 and 143 ng/ml for HNP-1, -2 and -3, respectively.

1. Introduction

Human neutrophil peptides (HNP-1, -2, -3 and -4) are antimicrobial molecules, belonging to the alpha-subfamily of human defensins and are stored in the granules of neutrophils. Two other alpha-defensins are localized in Paneth cell granules and are named human alpha-defensin 5 and 6 (HD-5 and -6) [1]. All alpha-defensins contain six cysteine amino acid residues, linked in three disulfide bonds, as shown in Table 1. HNPs make up about 5–7% of the total cellular protein content in the neutrophils and are the most abundant proteins (30–50%) in the azurophil granules [2–4]. Compared to HNPs 1–3, HNP-4 shows less amino acid sequence homology and a 100-fold lower concentration in the neutrophils [2].

The clinical relevance of HNPs 1–3 has been studied relatively extensively, whereas less is known about the other alpha-defensins, partly due to the lack of available reference materials [1,5]. In these studies, elevated concentrations of HNPs 1–3 in various matrices have been related to different types of diseases, suggesting high diagnostic potential. Infections have shown to result in up-regulated levels of HNPs 1–3 in plasma, blood, and other body fluids [3,6,7]. Furthermore, several studies have shown an increased concentration of HNPs 1–3 in plasma, blood, serum or bronchoalveolar lavage fluid (BALF) of patients with various types of lung diseases [3,8–12]. Besides infections and lung diseases, raised HNP levels have been related to gastro-intestinal diseases like Crohn's disease, ulcerative colitis and gastric or colorectal cancer [13–19]. Additionally, HNPs 1–3 have been related to renal cell carcinoma [20,21] and have been found up-regulated in saliva samples of patients with oral diseases [22–24].

To further explore the role of HNP-1, -2 and -3 as diagnostic markers for specific diseases, a widely applicable analytical assay would be beneficial. Previous quantification of HNPs 1–3 has mainly been performed by radio- or enzyme immunoassays. How-

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Table 1
Characteristics of human alpha-defensins [5].

Peptide	MW (Da)	Aa	Sequence	Disulfide bridges
HNP-1	3442.1	30	ACYCRIPACIAGERRYGTCIYQGRLWAFCC	2–30; 4–19; 9–29
HNP-2	3371.1	29	CYCRIPACIAGERRYGTCIYQGRLWAFCC	1–29; 3–18; 8–28
HNP-3	3486.1	30	DCYCRIPACIAGERRYGTCIYQGRLWAFCC	2–30; 4–19; 9–29
HNP-4	3824.6	34	VCSCRLVFCRRTELRVGNCLIGGVSFTYCCTRVD	2–30; 4–19; 9–29
HD-5	3582.2	32	ATCYCRTGRCATRESLSGVCEISGRLYRLCCR	3–31; 5–20; 10–30
HD-6	3708.3	32	AFTCHCRRSCYSTEYSYGTCTVMGINHRFCCL	4–31; 6–20; 10–30

ever, the specificity of these techniques does not allow distinction between the homologous HNP subtypes. The group of Mizukawa et al. [22–24] reported the use of LC with UV detection for the quantification of HNP-1 in saliva. Nevertheless, it remained unclear whether interference from other defensins was actually absent. The high specificity of mass spectrometric detection offers the benefit to enable separate quantification of HNP-1, -2 and -3. However, only one quantitative assay for HNP-1, -2 and -3 with MS detection has been reported [25], using LC–MS for saliva samples.

Here, an LC–MS/MS assay is presented as the first method to allow simultaneous quantification of the individual concentrations of HNP-1, -2 and -3 in human plasma and serum. A simple protein precipitation step is sufficient for sample purification, enabling rapid sample preparation and facilitating high sample throughput. Combined with the applicability of the assay as shown in serum samples taken from intensive care patients, this assay will provide the opportunity to measure large sets of clinical samples to clarify the diagnostic value of these antimicrobial peptides.

2. Experimental

2.1. Chemicals and reagents

Human neutrophil peptide-1, -2 and -3 as well as human alpha-defensin 5 were obtained from Peptanova (Sandhausen, Germany) in portions containing exactly 0.1 mg. Acetonitrile (gradient grade), methanol (HPLC grade) and LC–MS grade water were from Biosolve (Valkenswaard, The Netherlands), formic acid (p.a.) from Merck (Darmstadt, Germany) and trifluoroacetic acid (99.5%) from Acros Organics (Geel, Belgium). Blank human plasma and serum were obtained from the Sanquin Bloodbank (Utrecht, The Netherlands).

2.2. Instruments

The LC–MS/MS analyses were performed using an Accela high-speed chromatographic system coupled to a TSQ Quantum Ultra triple quadrupole mass spectrometer equipped with a heated electrospray ionization (H-ESI) probe (all from Thermo Fischer Scientific, San Jose, CA, USA). The analytical column, a Symmetry 300 C_{18}, 50 mm × 2.1 mm I.D., with 3.5 μm particle size (Waters Chromatography, Milford, MA, USA) was protected by a Polaris C_{18}-A guard column, 10 mm × 2.0 mm I.D. with 3 μm particle size (Varian Inc., Palo Alto, CA, USA).

2.3. LC–MS/MS conditions

Formic acid (0.25%, v/v) in water was used as eluent A and methanol as eluent B. The flow rate was set to 300 μl/min and column temperature to 30 °C, while autosampler temperature was maintained at 5 °C. During 3 min after injection eluent B was increased from 20 to 30%, followed by an increase to 90% in 1.8 min, both via a linear gradient. Eluent B was maintained at 90% for 0.7 min, followed by a rapid return to the initial conditions (25% B), which were kept during 1 min for re-equilibration. Total run time was 6.5 min.

The analytes were detected with quasi-multiple reaction monitoring (MRM) in the positive ion mode, measuring the quaternary charged molecular ion as both precursor and product at m/z 861.4 for HNP-1, 843.6 for HNP-2 and 872.4 for HNP-3 and the quintuple charged molecular ion at m/z 717.3 for HD-5, all with a collision pressure of 2.1 mTorr and collision energy of −20 eV. Further optimized conditions were nitrogen sheath, ion sweep and auxiliary gasses at 50, 4 and 55 arbitrary units (AU), respectively, ion tube temperature at 320 °C, spray voltage at 4.5 kV and vaporizer temperature at 100 °C. Unit resolution (0.70 FWHM) was used for Q1 and 1.40 FWHM for Q3 to obtain optimal signal–noise (S/N) ratios.

2.4. Preparation of standards and quality control samples

For all analytes, two separate portions of exactly 0.1 mg were dissolved in methanol/0.1% formic acid in water, 1:1 (v/v), to obtain stock solutions of exactly 100 μg/ml. Stock solutions of each analyte were combined and diluted to obtain a working solution of 25 μg/ml HNP-1, -2 and -3 for preparation of the calibration standards. Other stock solutions were combined and diluted to 20 μg/ml for preparation of the quality control (QC) samples. The internal standard (IS) working solution contained 2.5 μg/ml HD-5 in methanol/0.1% formic acid in water, 1:1 (v/v). Stock solutions were stored at −80 °C and working solutions at −30 °C.

One batch of human plasma with relatively low amounts of endogenous HNPs was selected for the preparation of calibration standards. HNP-1 and -3 concentrations in this batch were exactly quantified, using standards in analyte-free bovine plasma and a high concentration of HNP-2 as IS (1 μg/ml) to correct for species-specific matrix variations. Similarly, HNP-2 concentrations were determined with HNP-1 as IS (500 ng/ml). Standards were prepared by serial dilution in concentrations from 5 to 1000 ng/ml above the endogenous concentration. QC samples were prepared by serial dilution in other batches of human plasma and serum. The low QC (LQC) concentration in plasma was 12.5 ng/ml above the endogenous concentration for HNP-1 and -3 and 75 ng/ml above the endogenous concentration for HNP-2. LQC concentrations in serum were 50 ng/ml above the endogenous concentration for all peptides. Mid QC (MQC) and high QC (HQC) concentrations were 250 ng/ml and 800 ng/ml above the endogenous concentrations, respectively, for all peptides in both matrices. Furthermore, plasma and serum were spiked at 5000 ng/ml and measured after 5-fold dilution with the same human plasma batch as used for the calibration standards as the upper limit of quantification (ULQ).

2.5. Sample preparation

Peptides were extracted from plasma and serum by protein precipitation with 1% TFA (v/v) in acetonitrile. To a 100 μl aliquot of serum or plasma, 10 μl IS working solution and 200 μl precipitation solvent were added. The samples were vortex-mixed for 10 s and centrifuged at room temperature (5 min; 14,000 × g). The supernatant was then evaporated under a stream of nitrogen at 30 °C. After reconstitution of the residue in 400 μl of acetonitrile/water/formic acid (25/75/0.25, v/v), the sample was vortex-mixed (10 s) and centrifuged (10 min; 14,000 × g; 10 °C),

before the clear solution was transferred into glass vials. A volume of 10 μl was injected into the LC–MS system.

2.6. Method validation

Validation was based on the FDA guidelines for Bioanalytical Method Validation [26].

2.6.1. Linearity

Ten calibration standards were prepared by serial dilution in concentrations of 0; 5; 10; 20; 50; 100; 200; 400; 700 and 1000 ng/ml above the endogenous concentration of HNP-1, -2 and -3, quantified as 7.4, 41.3 and 5.8 ng/ml, respectively. All standards were analyzed in each run, calculating target peptide: IS ratios of the peak area for each concentration level. Standard curves were constructed by least-squares linear regression analysis using a weighting factor of $1/x^2$ (with x as the concentration in ng/ml).

2.6.2. Precision and accuracy

Precisions and accuracies of the method for plasma and serum samples were determined by analysis of samples with endogenous concentrations and QC samples spiked at three different concentration levels in three separate runs ($n = 18$ at each level). Furthermore, one run contained plasma and serum spiked above the ULQ level at 5000 ng/ml, analyzed after 5-fold dilution with the same human plasma batch as used for the calibration standards to validate dilution of the samples. The accuracies of the recovered analytes were calculated as [((overall measured concentration–initial concentration)/added concentration) × 100%]. Intra- and inter-assay precisions were expressed as relative standard deviations (RSD).

2.6.3. Selectivity

Human serum and plasma samples from six different sources were spiked at concentrations of 100 ng/ml and analyzed in triplicate. The plasma samples consisted of samples with EDTA, lithium heparine or citrate–phosphate–dextrose as anti-coagulant. For all sample batches, endogenous concentrations were measured by analysis in triplicate and recoveries were expressed as the difference between the average measured concentration in the spiked sample and the initial concentration. Furthermore, variations within and in-between the various batches were calculated.

2.6.4. Recovery and ion suppression

The extraction recovery was determined by comparing the peak areas of plasma and serum samples spiked before precipitation with samples spiked after precipitation, corrected for the peak areas of the endogenous peptides. All samples were analyzed in triplicate at three different concentrations (LQC, MQC and HQC). Furthermore, the ion suppressive effect of the matrix was assessed by post-column infusion of the peptides, determining the effect on their response after injection of blank matrix.

2.6.5. Stability

The stabilities of the peptides under different conditions were assessed at various concentrations during all stages of the method. In both plasma and serum, stabilities of the peptides were assessed after 2.5 h at room temperature, after three freeze-thaw cycles (−80 °C) and after different periods of storage at −80 °C, by comparison to the response of freshly prepared samples. Other stability experiments included the examination of the stabilities in the autosampler after 7 days storage and in the stock and working solutions. Stabilities of the peptides in the working solutions were assessed after 24 h at room temperature and one month storage at −30 °C, whereas stabilities of the peptides in the stock solutions were assessed after six months storage at −80 °C. Deviations were calculated by comparing MS response ratios to freshly prepared samples at identical concentrations.

All stability tests were performed in triplicate and at three different concentration levels (LQC, MQC and HQC). The analytes were considered stable when 85–115% of the initial concentration was found.

2.7. Analysis of serum samples from intensive care patients

HNP-1, -2 and -3 concentrations were quantified in 24 serum samples obtained from 11 different patients at the intensive care unit. One serum sample of each patient was collected at 6.00 p.m., while additional samples of some patients were drawn on different days. Samples with concentrations beyond the ULQ of the assay were re-analyzed after 2- or 5-fold dilution of the sample with plasma from the same batch as used for preparation of the calibration standards. QC samples of all concentration levels were measured along with the patient samples to monitor the analytical performance.

3. Results and discussion

3.1. Method development

3.1.1. Sample pre-treatment

The protein precipitation procedure offered rapid sample preparation, facilitating high sample throughput. It furthermore provided better recoveries, especially for HNP-2, than solid-phase extraction (SPE) on different types of reversed-phase cartridges, although SPE is most commonly applied for sample clean-up of peptides from complex matrices [27]. Acidification of the organic solvent was required to prevent co-precipitation of the analytes. Addition of 200 μl instead of 250 μl acetonitrile resulted in substantially clearer extracts. Furthermore, evaporation of the supernatant appeared beneficial as interferences which were insoluble in the reconstitution solvent could be removed after reconstitution and subsequent centrifugation. However, as no solid precipitate was formed after reconstitution, addition of a larger volume of reconstitution solvent (400 μl) was necessary to prevent injection of insoluble particles into the LC–MS/MS system. Moreover, this dilution did not affect the assay's sensitivity, probably due to reduced ion suppressive effects.

3.1.2. LC–MS/MS analysis

ESI-MS spectra of HNP-1, -2 and -3 and the IS are presented in Fig. 1, showing the quaternary charged protonated molecules as most abundant for HNPs 1–3 and the quintuple charged ion for HD-5. Fragmentation of the peptides by collision-induced dissociation (CID) required high collision energies and resulted in very poor signal intensities of the various product ions, probably due to the disulfide cross-linking. Therefore, lower collision energies were applied and the same m/z value as selected for CID was selected as "product ion" in the MRM mode for all defensins. This "quasi"-MRM approach resulted in higher peak intensities and lower background noise in comparison to single ion monitoring (SIM), as the applied collision energy could reduce interferences from compounds with similar molecular masses (Fig. 2).

The applied gradient with an initially slowly increasing percentage of organic solvent was required to reduce ion suppressive effects from matrix components, especially on the IS response. The rapid increase in organic solvent hereafter was needed to provide close elution of the analytes and IS without severe peak broadening of the IS. Both HD-5 and HNPs 1–3 eluted within 4.3–4.6 min and were sufficiently separated from possible interferences. Because HNPs 1–3 share almost similar amino acid sequences, separation

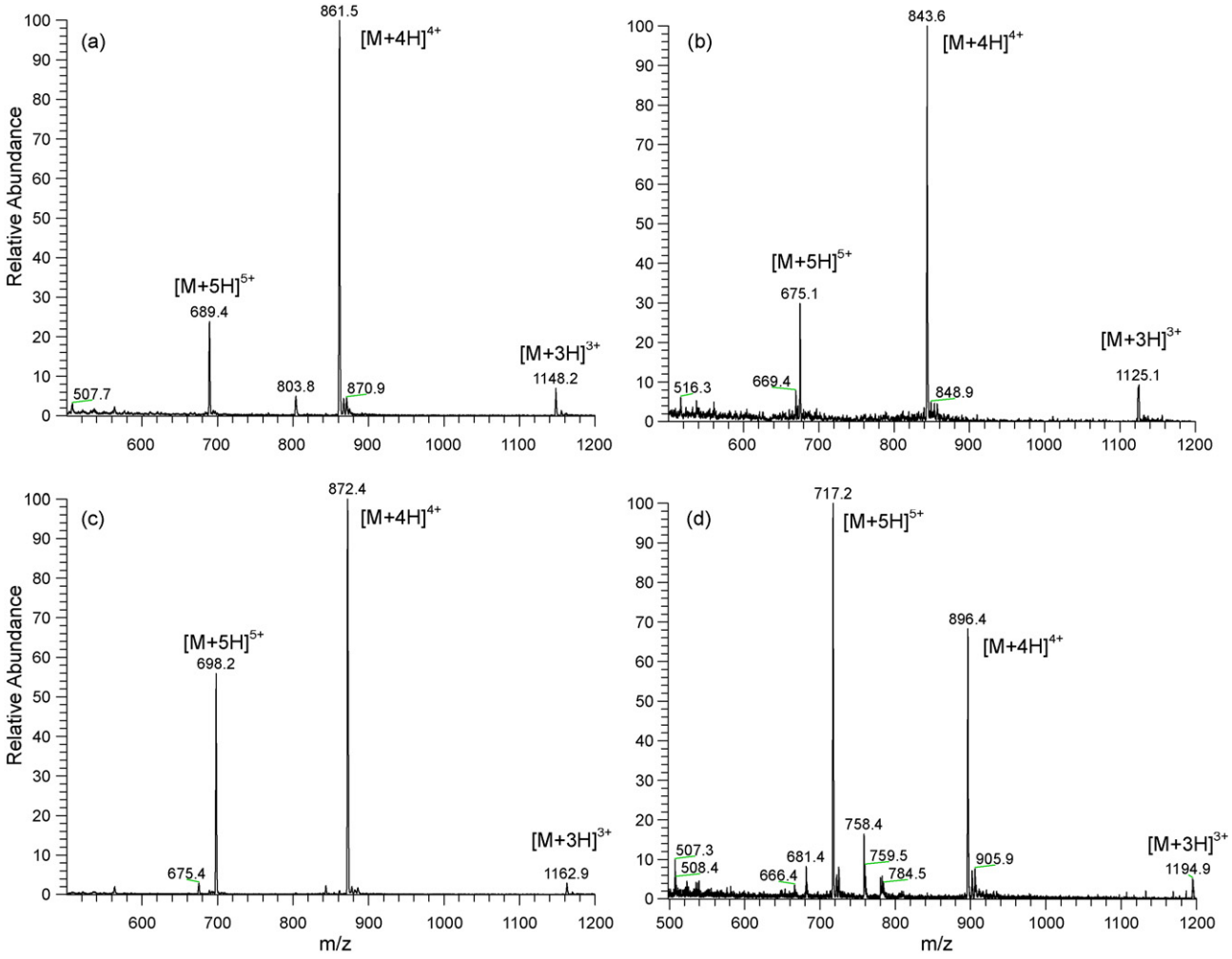

Fig. 1. ESI-MS spectra of a) HNP-1; b) HNP-2; c) HNP-3 and d) HD-5.

of these analytes was complicated, but appeared unnecessary as observed cross-interferences between the HNP-subtypes at HQC levels were below 10% of the LLOQ response. Moreover, close elution of the analytes with the IS was specifically required since the IS appeared more easily affected by ion suppressive effects of other matrix components. Ideally, an isotope-labeled IS would therefore be recommended.

Examples of quasi-MRM chromatograms of non-spiked serum and serum spiked at the LQC concentration are shown in Fig. 3.

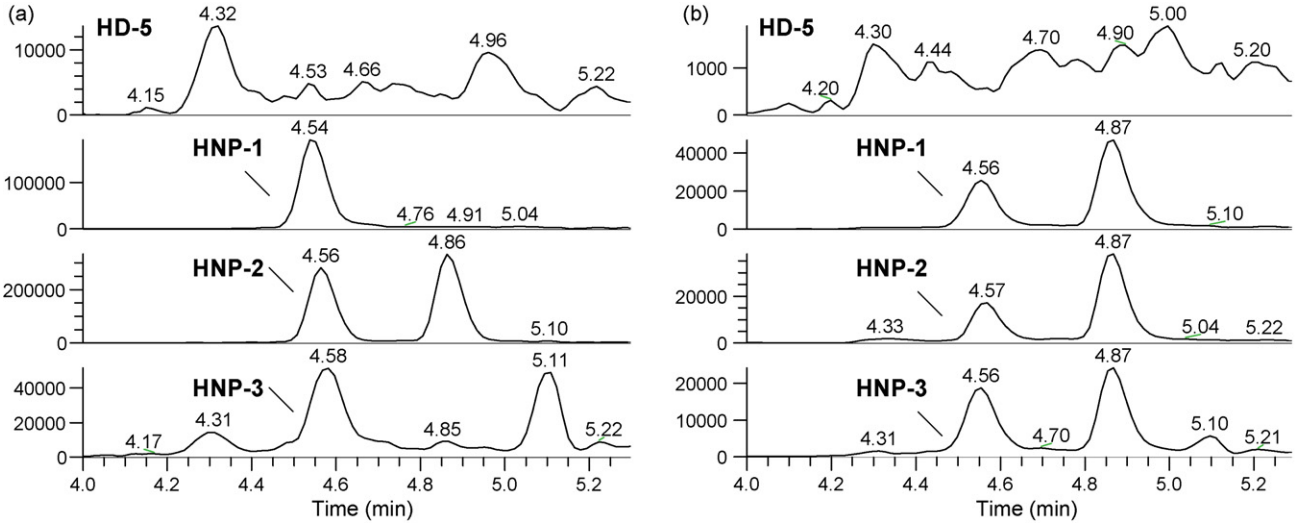

Fig. 2. Comparison of a) a "quasi"-MRM and b) a SIM chromatogram obtained for a non-spiked serum sample.

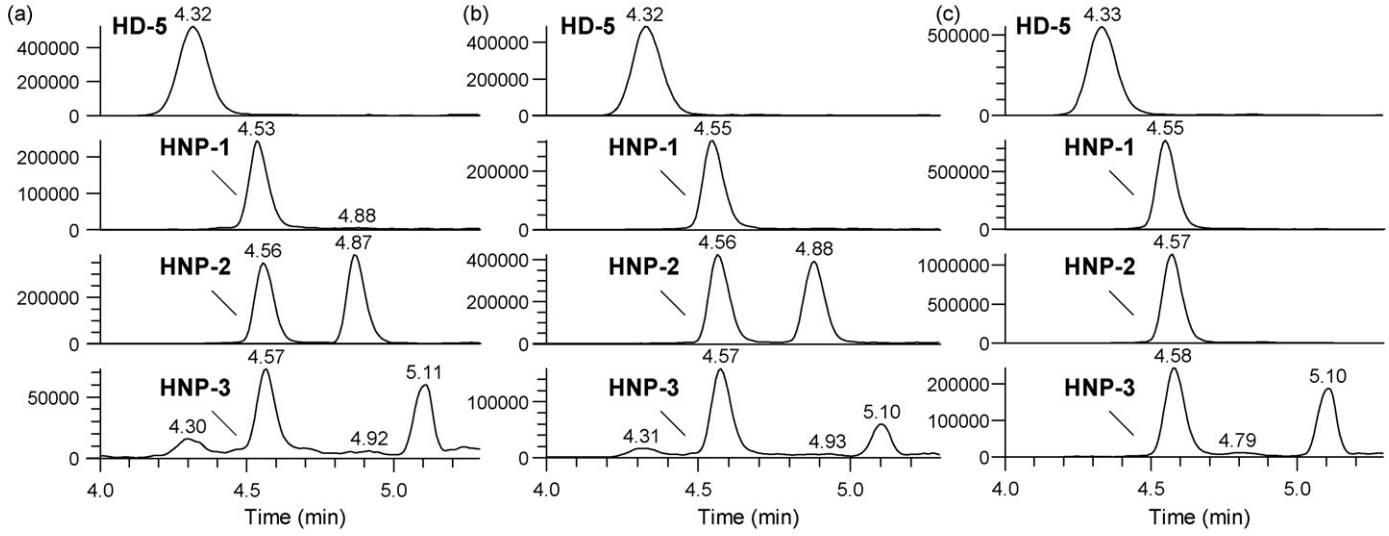

Fig. 3. MRM chromatograms of a) a non-spiked serum sample with IS; b) serum spiked at LQC level and c) serum of an IC-patient.

3.2. Method validation

3.2.1. Linearity

The method was linear from 5 to 1000 ng/ml for HNP-1, -2 and -3. The mean determination coefficients (R^2; $n = 3$) were 0.9962 ± 0.002, 0.9887 ± 0.004 and 0.9947 ± 0.004 for HNP-1, -2 and -3, respectively. The mean y-intercepts were 0.0048 ± 0.01, 0.0139 ± 0.06 and 0.0001 ± 0.004 for HNP-1, -2 and -3, respectively and the mean slopes (ml/ng) were 0.0027 ± 0.0001 for HNP-1, 0.0028 ± 0.0002 for HNP-2 and 0.0027 ± 0.0002 for HNP-3.

3.2.2. Accuracy and precision

The assay performance data in human plasma and serum are presented in Table 2. Intra- and inter-assay precisions at the lowest concentration (6.4 ng/ml HNP-1 in plasma) were 14.8 and 19.1% respectively, indicating the lower limit of quantification (LLOQ). While HNP-2 and -3 showed comparable signal intensities and response variations as did HNP-1 and results in serum were not different from results in plasma, we suggest this LLOQ for all three peptides in plasma as well as serum. At all other concentration levels, intra- and inter-assay precision as well as deviations from the added concentration for plasma and serum samples were below 11.2, 14.7 and 15.0%, respectively.

Furthermore, concentrations of samples spiked above the ULQ at 5000 ng/ml were precisely quantified after 5-fold dilution, although showing a negative deviation from the expected concentrations for all peptides. Nonetheless, these results were obtained from one single run and deviations were within 15% in both matrices for HNP-2, which is most likely to require sample dilution.

3.2.3. Selectivity

Endogenous concentrations in six different batches of human plasma varied between 4 and 42 ng/ml for HNP-1, 33–127 ng/ml for HNP-2 and 5–19 ng/ml for HNP-3. Mean differences between blank and spiked human plasma samples were 98.3 ± 5.9, 101.3 ± 8.2 and 90.9 ± 5.7 ng/ml, for HNP-1, -2 and -3, respectively. Variations in measured concentrations within one batch were below 14% for all peptides. For serum samples, average differences between blank and spiked samples were 88.2 ± 4.8 ng/ml for HNP-1, 98.5 ± 8.9 ng/ml for HNP-2 and 94.2 ± 8.8 ng/ml for HNP-3 with endogenous concentrations in the different serum samples varying between 16 and 108 ng/ml, 113–267 ng/ml and 0–149 ng/ml for HNP-1, -2 and -3, respectively. Variations in measured concentrations within one batch were below 13% for all peptides. These analyses showed that accurate and precise quantification is possible in various human matrices. Furthermore, endogenous HNP-1, -2 and -3 concentrations appeared to be generally lower in plasma samples.

3.2.4. Recovery and ion suppression

Recoveries at LQC concentrations in plasma and serum, as well as at MQC concentrations in serum resulted in small differences between the sample and control areas, due to the response of the endogenous peptides and varied from 90.7 to 128.3%. Recovery values for plasma samples spiked at HQC concentrations were 86.7 ± 11.5, 86.8 ± 12.1 and 85.2 ± 10.8% for HNP-1, -2 and -3, respectively, while similar recoveries were obtained for serum samples: 85.4 ± 10.9, 90.2 ± 10.3 and 85.2 ± 11.3% for HNP-1, -2 and -3, respectively. Recovery of the IS was 88 ± 14% for both matrices. With the applied gradient ion suppressive effects were below 20% for all peptides. Complete recovery data as well as an example of the ion suppressive effects of the matrix on the analyte responses during a chromatographic run are presented in Table 3 and Fig. 4, respectively.

3.2.5. Stability

Working solutions were stable during one-month storage at −30 °C and 4 h storage at room temperature. Stock solutions were stable during six months storage at −80 °C. Although HD-5 initially appeared unstable in the reconstitution solvent, this was not a problem in the plasma and serum extracts. Accurate quantification of the final extracts after one week storage at 5 °C with freshly prepared standards could be achieved, as well as with calibration standards stored for a similar period of time.

In contrast to the potential biomarker peptides derived after proteolytic cleavage of serum or plasma proteins [28,29], HNP-1, -2 and -3 appeared resistant to protease activity and were stable in their biological environment. HNP-1, -2 and -3 did not show any instability in either plasma or serum after three freeze-thaw cycles (−80 °C), 2.5 h storage at room temperature and two months storage at −80 °C. This can be clinically relevant as it improves the usefulness of HNPs 1–3 for diagnostic purposes by reducing non-disease related variations in HNP-1, -2 and -3 concentrations. Furthermore, no differences in stability were observed between serum and plasma samples. The stability data for serum samples are presented in Table 4.

Table 2
Assay performance data for HNP-1, -2 and -3 in human plasma and serum.

		Sample	Average measured concentration (ng/ml)[a]	% Recovery	Intra-assay precision (%)	Inter-assay precision (%)
Plasma	HNP-1	Blank	6.4 ± 1.2		14.8	19.1
		LQC	18.7 ± 2.7	−1.8	6.2	14.7
		MQC	239.8 ± 25.2	−6.6	8.7	10.5
		HQC	804.2 ± 82.1	−0.3	10.0	10.2
		ULQ[b]	836.8 ± 29.1	−16.3	3.5	
	HNP-2	Blank	56.6 ± 6.0		9.8	10.5
		LQC	142.8 ± 12.1	15.0	7.5	8.5
		MQC	312.4 ± 34.9	2.3	8.4	11.2
		HQC	862.2 ± 96.5	0.7	10.4	11.2
		ULQ[b]	893.9 ± 31.0	−10.6	3.5	
	HNP-3	Blank	9.1 ± 1.0		10.1	10.7
		LQC	21.1 ± 3.1	−4.5	11.2	14.7
		MQC	240.4 ± 22.8	−7.5	8.6	9.5
		HQC	800.7 ± 93.7	−1.1	10.3	11.7
		ULQ[b]	856.8 ± 28.8	−14.3	3.4	
Serum	HNP-1	Blank	92.1 ± 9.1		5.6	9.9
		LQC	145.9 ± 18.4	7.5	5.3	12.6
		MQC	343.2 ± 28.1	0.4	4.7	8.2
		HQC	869.8 ± 66.7	−2.8	5.8	7.7
		ULQ[b]	899.4 ± 49.0	−10.1	5.4	
	HNP-2	Blank	105.9 ± 10.9		6.5	10.3
		LQC	163.3 ± 15.7	14.8	6.7	9.6
		MQC	368.0 ± 29.7	4.8	5.1	8.1
		HQC	908.0 ± 58.5	0.3	6.0	6.4
		ULQ[b]	884.5 ± 41.4	−11.5	4.7	
	HNP-3	Blank	24.9 ± 1.8		6.7	7.3
		LQC	74.0 ± 4.2	−1.7	5.9	5.7
		MQC	268.3 ± 14.4	−2.7	5.2	5.4
		HQC	794.3 ± 73.4	−3.8	5.5	9.2
		ULQ[b]	848.9 ± 51.3	−15.1	6.0	

[a] Measured concentrations include endogenous and spiked peptides. Expected LQC concentrations in plasma were 12.5 ng/ml above the "blank" concentration for HNP-1 and -3 and 75 ng/ml for HNP-2. In serum, LQC samples were spiked with 50 ng/ml. MQC and HQC samples were spiked with 250 and 800 ng/ml, respectively in both matrices.
[b] ULQ samples were spiked at 5 μg/ml in both matrices and measured after 5-fold dilution with blank human plasma from the same batch as used for preparation of the calibration standards.

3.3. Internal standard and matrix selection

HD-5 was selected as IS, because of its comparable molecular weight and disulfide cross-linking. Although the IS showed a different chromatographic behavior as the HNPs and was more easily affected by ion suppression, the IS could sufficiently correct for response variations in the human plasma and serum samples. Furthermore, no interference from endogenous HD-5 was observed in the various tested serum and plasma samples. However, quantification in human plasma or serum, using standards in analyte-free bovine or mouse plasma, was not possible because the IS could not correct for species-specific matrix differences. Therefore, human

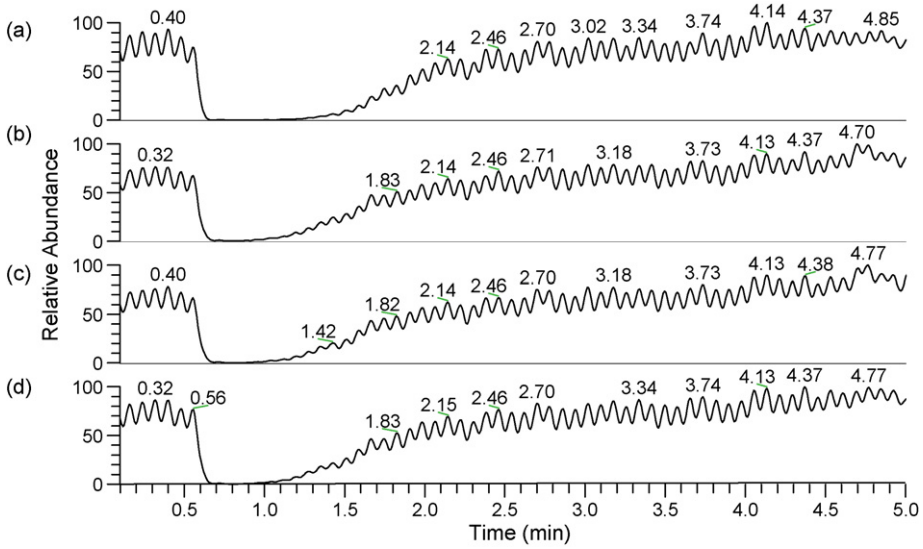

Fig. 4. Ion suppressive effects of a blank serum sample on a) HD-5; b) HNP-1; c) HNP-2 and d) HNP-3. A similar chromatogram was obtained for human plasma.

Table 3
Recovery data of HNP-1, -2, -3 and HD-5 from serum and plasma at three different concentration levels ($n = 3$).

	LQC	MQC	HQC
Plasma			
HNP-1	102.4 ± 10.1	95.1 ± 6.3	86.7 ± 11.5
HNP-2	121.4 ± 8.6	104.7 ± 5.5	86.8 ± 12.1
HNP-3	108.7 ± 15.3	94.6 ± 10.6	85.2 ± 10.8
HD-5			88.2 ± 12.3
Serum			
HNP-1	106.5 ± 7.7	112.5 ± 10.8	85.4 ± 10.9
HNP-2	128.3 ± 8.2	121.7 ± 10.3	90.2 ± 10.3
HNP-3	90.7 ± 8.6	114.7 ± 11.4	85.2 ± 11.3
HD-5			87.8 ± 16.0

plasma with low endogenous levels of the analytes was selected for preparation of the calibration standards, allowing accurate quantification of HNP-1, -2 and -3 concentrations in various human plasma and serum batches. Nevertheless, replacement of the pre-column after ±200 injections appeared necessary as decreased column performance contributed to increased ion suppression and response variation due to different effects on analytes and IS. These problems are therefore likely to be reduced when a more analogous IS is used, particularly because the above mentioned response variations within plasma samples from different species were not observed when one of the analytes was used as IS to quantify the two remaining peptides. Therefore, we recommend synthesis of a stable isotope-labeled analog of HNPs 1–3, which was too costly in this stage of method development, if the assay is applied for routine use.

Table 4
Stability data of HNP-1, -2 and -3 in serum and serum extracts.

Peptide	Matrix	Condition	LQC		MQC		HQC	
			% DEV	RSD	% DEV	RSD	% DEV	RSD
HNP-1	Serum	Freeze-thaw	8.7	6.4	0.2	13.4	9.9	11.8
HNP-2			−3.5	9.7	−4.6	13.9	7.0	12.0
HNP-3			−0.3	12.9	−6.6	13.6	9.1	13.2
HNP-1	Serum	Short-term 2.5 h RT	−1.8	5.6	5.5	5.3	−1.8	8.4
HNP-2			0.7	5.7	2.4	7.0	−2.7	7.3
HNP-3			−0.4	5.9	7.0	6.7	−2.1	8.3
HNP-1	Serum	Long-term Two months −80 °C	19.2	8.6	8.7	5.2	8.2	6.7
HNP-2			0.2	6.3	−8.4	4.9	−5.6	4.4
HNP-3			6.0	5.6	1.5	6.8	−2.7	9.4
HNP-1	Final extract	Autosampler 5 °C; 7 days	−6.9	4.9	−0.1	8.3	−9.7	11.4
HNP-2			−4.1	5.7	−0.3	10.0	−9.2	10.2
HNP-3			−2.4	5.5	2.1	10.0	−10.3	11.6

Table 5
Measured concentrations of HNP-1, -2 and -3 in serum samples from intensive care patients.

Individual	Days between first sample	Concentration			
		CRP (mg/l)	HNP-1 (ng/ml)	HNP-2 (ng/ml)	HNP-3 (ng/ml)
#1	–	26.2	94.9	187.7	35.1
	1	110.5	105.4	214.9	42.8
	2	150.4	147.6	331.9	61.6
	10	7.9	213.9	397.1	83.9
	11	7.2	91.4	245.4	32.2
#2	–	135.8	568.2	659.2	358.0
	1	137.2	474.9	487.8	281.0
	8	NM	579.0	587.0	376.3
	10	84.6	368.2	409.7	236.0
#3	–	300.8	1814.6	2300.3	383.3
	6	NM	332.4	1143.9	127.9
	8	20.6	343.1	1144.7	125.3
#4	–	26.9	282.6	332.7	0.0
	1	23.7	289.8	582.6	4.0
#5	–	221.3	22.0	197.5	18.2
	2	172.7	42.3	432.0	31.1
#6	–	307.5	152.7	566.0	465.0
	1	220.6	25.3	284.9	94.3
#7	–	288.8	576.0	654.9	100.3
	2	320.1	679.0	857.4	137.2
#8	–	49.7	64.3	292.9	14.3
#9	–	54.8	1237.4	924.7	173.3
#10	–	176	84.8	207.9	187.2
#11	–	19.8	105.9	239.8	72.6

NM: not measured.

Without the availability of a stable isotope-labeled IS, application of a human matrix for preparation of the standards and QC samples is required. For preparation of the calibration standards, the endogenous concentrations of HNPs 1–3 are preferably as low as possible, and we recommend endogenous concentrations below 15 ng/ml for HNP-1, below 10 ng/ml for HNP-3 and below 80 ng/ml for HNP-2. These concentrations can be exactly quantified for two of the analytes with the remaining peptide as IS, using standards in an analyte-free matrix such as bovine or mouse plasma. Especially in human plasma, concentrations can be expected below the above mentioned recommended values, observed for HNP-1 and -3 in five out of six batches of plasma used for selectivity assessment, as well as in four batches for HNP-2.

3.4. Analysis of serum samples from intensive care patients

Measured concentrations for HNP-1, -2 and -3 are summarized in Table 5 along with concentrations of C-reactive protein (CRP), measured in the same samples. The CRP levels serve as an indication for inflammation, which has been reported to cause up-regulated levels of HNPs 1–3 in biological fluids [3,6,7]. However, no clear correlation between CRP and HNP-1, -2 and -3 concentrations were observed and none of the antimicrobial peptides seemed specifically increased in all serum samples. HNP-2 showed the highest concentrations in almost all serum samples (188–2300; average 570 ng/ml), whereas HNP-3 usually appeared in the lowest amount (0–465; average 143 ng/ml). Only for HNP-3, concentrations below the LLOQ of the assay were measured in two serum samples. Measured concentrations of HNP-1 ranged from 22 to 1815 ng/ml with an average of 362 ng/ml. These analyses show the applicability of the assay and the validated concentration range (5–1000 ng/ml) for quantification of clinical samples.

HNP-1, -2 and -3 concentrations could only be compared with the total amount of HNPs 1–3, measured by radio- and immunoassays. Reported HNPs 1–3 serum concentrations measured with RIA [9] were around 250 ng/ml for controls, while between 500 and 1750 ng/ml in patients with various lung diseases. In another study, HNPs 1–3 concentrations in serum were measured with ELISA [16] and reported as ±7 ng/ml in controls and increased in colon cancer patients with a median concentration around 15–29 ng/ml. When comparing the total amount of HNP-1, -2 and -3 as measured with the developed assay in serum from IC patients (average 1076 ng/ml), these levels comply with the up-regulated concentrations found in the study of Mukae et al., whereas the total measured amount in the serum batches used for the selectivity assessment (average 309 ng/ml) are comparable with the concentrations of the healthy controls in the same study [9]. More studies have reported HNPs 1–3 concentrations in plasma, which were for healthy controls around 200–400 ng/ml when measured with RIA [3,6,8,10–12,19] and around 40–100 ng/ml when measured with ELISA [7,14,15]. Our results measured in different plasma batches used for selectivity assessment were between 40 and 175 ng/ml and seem comparable with the concentrations measured with the ELISA methods.

4. Conclusion

An LC–MS/MS method requiring simple sample preparation has been developed for the quantitative analysis of individual HNP-1, -2 and -3 concentrations in human plasma and serum. In this study, the applicability of the method has been shown in the analysis of serum samples from IC patients. Furthermore, the assay might be suitable as a template for quantitative measurements in other biological fluids, as urine or saliva. Therefore, the assay is likely to aid in the further exploration of the diagnostic value of these antimicrobial peptides and will firstly be applied to explore whether HNP-1, -2 and -3 play a role as serum markers of colon cancer.

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