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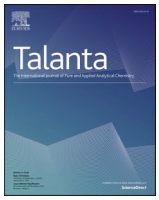

Development of a high-throughput liquid chromatography-tandem mass spectrometry platform for the determination of intact natriuretic peptides in human plasma

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ABSTRACT

We present an innovative, reliable, and antibody-free analytical method to determine multiple intact natriuretic peptides in human plasma. These biomolecules are routinely used to confirm the diagnosis and monitor the evolution of heart failure, so that their determination is essential to improve diagnosis and monitor the efficacy of treatment. However, common immunoassay kits suffer from main limitations due to high cross-reactivity with structurally similar species. In our method, we pre-treated the sample by combining salting-out with ammonium sulfate with microextraction by packed sorbent technique. Analyses were then carried out by ultra-high performance liquid chromatography-electrospray ionization-tandem mass spectrometry. The use of 3-nitrobenzyl alcohol as a supercharger reagent enhanced the ESI ionization and improved the signal-to-noise ratio. The analytical protocol showed good linearity over one order of magnitude, recovery in the range of 94–105 %, and good intra- and inter-day reproducibility (RSD<20 %), and the presence of a matrix effect. Limits of detection were in the range of pg/mL for all peptides (0.2–20 pg/mL). Stability study in plasma samples demonstrated that proper protease inhibitors need to be included in blood collection tubes to avoid peptide degradation. Preliminary analyses on plasma samples from heart failure patients allow the quantification of ANP 1–28 as the most abundant species and the detection of ANP 5–28, BNP 1–32, and BNP 5–32. The method could be used to investigate how cross-reactivity issues among structurally similar species impact determinations by ELISA kits.

1. Introduction

Natriuretic peptides (NPs) are hormones synthesized from cardiomyocytes that play important roles in the regulation of blood pressure, natriuresis, and, more generally, cardiovascular homeostasis [1]. The NPs family consists of three biologically active peptides, namely atrial natriuretic peptide (ANP 1–28), B-type natriuretic peptide (BNP 1–32), and C-type natriuretic peptide (CNP 1–22, CNP). NPs have similar molecular structures that include a 17-amino acid ring and a disulfide bond, and they share eleven amino acids in the peptide chain (Fig. S1). These hormones are considered useful biomarkers for the diagnosis and prognosis assessment of heart failure (HF) [2], a chronic and progressive syndrome resulting in a compromised heart function and inefficient blood supply to body organs whose present prevalence (1–2 % in the adult population [3]) is expected to increase up to 3 % over the next decade in industrialized countries [4]. The high mortality, i.e. 17 % in 1-year from diagnosis and 50 % in 5 years, has remained unchanged in recent decades despite advances in treatment [4].

When non-specific HF symptoms (e.g., dyspnea) are observed, NPs are measured to confirm diagnosis [2]. NP plasma levels progressively increase with HF severity, and reach values close to hundreds of pg/mL for BNP 1–32 [2], tenths-hundreds of pg/mL for ANP 1–28 [5], and units to tenths of pg/mL for CNP [6] in patients with acute HF. Upon effective treatment NP plasma levels normalize and makes their reliable determination utterly important to assist physicians in managing the syndrome by proper and prompt pharmacological interventions [2].

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Nowadays, NPs are routinely quantified in blood or plasma by enzymatic immunoassays [7]. Despite their extensive use, immunoassays are prone to cross-reactions between the antibody and the antigen that hamper their standardization [8]. For instance, circulating truncated forms of NPs, which share the same molecular structure as the native NPs except for the loss of amino acid residues, may impact the specificity of the test, as discussed for BNP 1–32 [9]. Immunoassays employed for BNP 1–32 suffer from a lack of specificity due to the cross-reactions of the forms (e.g., BNP 3–32, BNP 4–32, and BNP 5–32) [8], produced by the rapid proteolytic degradation of BNP 1–32 in vivo [10]. Similarly, proteases degrade circulating ANP 1–28 into truncated forms known as ANP 4–28 (loss of three amino acid residues) and ANP 5–28 (loss of four amino acid residues) [11]. In 2015, Chavagnac et al. [12] compared 10 different commercial ELISA kits and showed a huge variability in the BNP 1–32 levels measured from different assays.

Analytical protocols based on liquid chromatography (LC) coupled with mass spectrometry detection (e.g., tandem mass spectrometry, MS/MS) have been suggested as an alternative to ELISA kit for their good selectivity and accuracy in the analysis of biomolecules in complex biological matrices [13]. In such cases, ultrafiltration is widely used to remove large proteins but the non-specific adsorption on membranes poses problems [14]. The cheap, widely available, and highly water-soluble ammonium sulfate is often used for salting out proteins [15] but it has poor specificity and often leads to co-precipitation of low molecular weight peptides and high-abundance proteins [16]. Microextraction by packed sorbent (MEPS) is a simple, rapid, and fully automated technique that requires a limited solvent volume and can be used as an additional clean-up step when analyzing metabolites in biological fluids [17]. Differently from the classical SPE, MEPS can reuse the same cartridge up to 50 times if the sample matrix is not extremely complex. Moreover, the elution of the retained analytes with only tenths of microliters of the solvent increases the analyte preconcentration factor [18]. All these aspects make MEPS an appealing technique to be combined with the salting-out procedure, as reported for blood, plasma, or urine samples [17].

The analysis of natriuretic peptides in plasma using LC-MS/MS instruments has been partially investigated in the literature. Most studies only determine BNP 1–32 and its degraded forms in plasma after performing reduction/alkylation and digestion steps [19,20], or by analyzing intact peptides [21–23], or by directly injecting standard solutions (by infusion) into the MS instrument [24]. Interestingly, in 2017, Torma et al. suggested a partial oxidation of the methionine residues of BNP 1–32 during sample treatment, highlighting the poor stability of the peptide [22]. Similar results have also been found by Niederkofler et al., who detected a small amount of methionine-oxidized BNP 3–32, BNP 4–32, and BNP 5–32 in plasma samples [21].

This work aimed at developing and validating an analytical workflow for the determination of an array of natriuretic peptides (i.e., ANP 1–28, ANP 4–28, ANP 5–28, BNP 1–32, BNP 3–32, BNP 5–32, and CNP) in human plasma. The method involved salting-out with ammonium sulfate combined with microextraction by packed sorbent (MEPS), followed by UHPLC-ESI-MS/MS.

2. Materials and methods

2.1. Chemicals

Lyophilized human atrial natriuretic peptide 1–28 (SLRRSSCFGGRMDRIGAQSGLGCNSFRY, Cys7-Cys23) (trifluoroacetate salt), human B-type natriuretic peptide 1–32 (SPKMVQGSGCFGRKMDRISSSSGLGCKVLRRH, Cys10-Cys26), and human C-type natriuretic peptide-22 (GLSKGCFGLKLDRIGSMSGLGC, Cys6-Cys22) (trifluoroacetate salt) at a purity of ≥95 % were purchased from Cayman Chemicals (Michigan, USA). Lyophilized human brain natriuretic peptide 3–32 (KMVQGSGCFGRKMDRISSSSGLGCKVLRRH, Cys8-Cys24), human brain natriuretic peptide 5–32 (VQGSGCFGRKMDRISSSSGLGCKVLRRH, Cys6-Cys22), human atrial natriuretic peptide 4–28 (RSSCFGGRMDRIGAQSGLGCNSFRY, Cys4-Cys28), and human atrial natriuretic peptide 5–28 (SSCFGGRMDRIGAQSGLGCNSFRY, Cys3-Cys19) at a purity of ≥95 % were purchased from Phoenix Pharmaceuticals Inc. (Burlingame, USA).

Rat atrial natriuretic peptide 1–28 (SLRRSSCFGGRIDRIGAQSGLGCNSFRY, Cys7-Cys23) at a purity of ≥95 % was purchased from Eurogentec (Seraing, Belgium) and used as internal standard (IS).

Acetonitrile (ACN), methanol (MeOH), and water were purchased from Merck Millipore (Milan, Italy) at LC-MS hypergrade (≥99.9 %) (LiChrosolv®). Formic acid (FA, for LC-MS LiChropur™, purity 98–100 %), trifluoroacetic acid (TFA, purity of ≥99 %), 3-nitrobenzyl alcohol suitable for mass spectrometry (3-NBA, purity of ≥99.5 %) dimethylsulfoxide (DMSO, purity of ≥99 %) ammonium sulfate (purity of ≥99 %), protease inhibitor cocktail P-8340 (dimethyl sulfoxide solution composed of AEBSF 104 mM, aprotinin 80 μM, bestatin 4 mM, E−64 1.4 mM, leupeptin 2 mM, and pepstatin A 1.5 mM), protease-free albumin from human serum (HSA, purity of ≥96 %), HPLC grade L-methionine (Met, purity of ≥98 %), and butylated hydroxytoluene (BHT, purity ≥99 %) were purchased from Merck (Milan, Italy).

2.2. Materials and equipment

All liquid solutions and plasma samples were stored in sterile protein LoBind polypropylene tubes from Eppendorf (Milan, Italy) to avoid secondary interactions with glass containers, as reported elsewhere [25].

Inj/Light® syringes were from Rays (Osimo, Italy). Phenex™-RC syringe filters (0.2 μm regenerate cellulose, 4 mm of diameter) were from Phenomenex (California, USA).

Amicon® Ultra centrifugal filters with a molecular weight cut-off of 10 kDa were from Merck Millipore (Milan, Italy). BD™ P100 blood collection tubes (2 mL) containing spray coated K2EDTA anticoagulant and protease inhibitor cocktail were from BD Biosciences (New Jersey, USA).

Prior to UHPLC injection, each sample or standard solution was stored in the autosampler in polypropylene vials (250 and 1000 μL volumes) purchased from Agilent Technologies (USA).

A VELP Scientifica ZX4 Advanced Vortex Mixer (Usmate, Italy), a Thermo Mixer HCM100-Pro (DLab Scientific, China), and a Hermle Z-326 K Centrifuge (Wehingen, Germany) were used for sample mixing and centrifugation, respectively.

The removable needle microextraction by packed sorbent (MEPS) syringe (250 μL) for HTA 300APlus (Thermo Scientific & Varian 8400 systems), MEPS Barrel Insert and Needles (BINs) assembly containing C2 or C18 sorbent materials were purchased from SGE Analytical Science (Melbourne, Australia).

The automated HT4000 Series Sample Prep workstation was purchased from HTA S.R.L. (Brescia, Italy).

2.3. UHPLC-ESI-MS/MS instrumentation and analysis

UHPLC-ESI-MS/MS analyses were performed using a 1290 Infinity II LC system coupled to a 6495 Triple Quadrupole mass spectrometer, equipped with a Jet Stream electrospray (ESI) ionization source (Agilent Technologies, USA). Chromatographic separation of target peptides was achieved using a Zorbax RRHD 300SB-C3 column (2.1 mm I.D. × 100 mm length, 1.8 μm particle size) and a gradient elution at 0.4 mL/min with a mobile phase consisting of formic acid (0.2 % v/v) and 3-NBA (0.01 % v/v) aqueous solution (A) and acetonitrile containing formic acid (0.2 % v/v and 3-NBA (0.01 % v/v) (B). The composition of the mobile phase changed over time as follows: 5 % (B) for 0.5 min, from 5 to 12 % (B) in 4.0 min, from 12 to 22 % (B) in 4.0 min, from 22 to 40 % (B) in 2.0 min, from 40 to 80 % (B) in 0.1 min, isocratic for 2.9 min, re-equilibration to initial conditions in 0.5 min, isocratic up to 20 min. The multisampler and the column compartment were set at 4 and 50 °C,

respectively. The injection volume was 5 μL.

The 6495 Triple Quadrupole mass spectrometer detector operated in ESI positive ionization and Multiple Reaction Monitoring (MRM) acquisition mode. For all the analytes, the ESI operation conditions were: drying gas temperature of 200 °C, drying gas flow of 18 L/min, nebulizer gas pressure of 50 psi, sheath gas temperature of 300 °C, sheath gas flow of 12 L min, capillary voltage of 3000 V and nozzle 500 V. Fragmentor voltage was fixed at 380 V and high- and low-pressure funnel voltages were set at 125 and 150 V, respectively. Optimization of the MRM method was obtained by acquiring full scan spectra ($350<m/z<1000$) and selecting a suitable precursor for each peptide and oxidative species, which were obtained from native peptides after 24 or 72 h of atmospheric exposure as described in paragraph 2.7. Then, product-ion scan spectra were acquired at various collision energies (from 0 to 25 eV) monitoring each peptide's most intense charge state to obtain the maximum sensitivity. The most intense transition was identified as the quantifier (Q), whereas two more transitions were labeled as qualifiers (q). For each transition, the dwell time was selected to

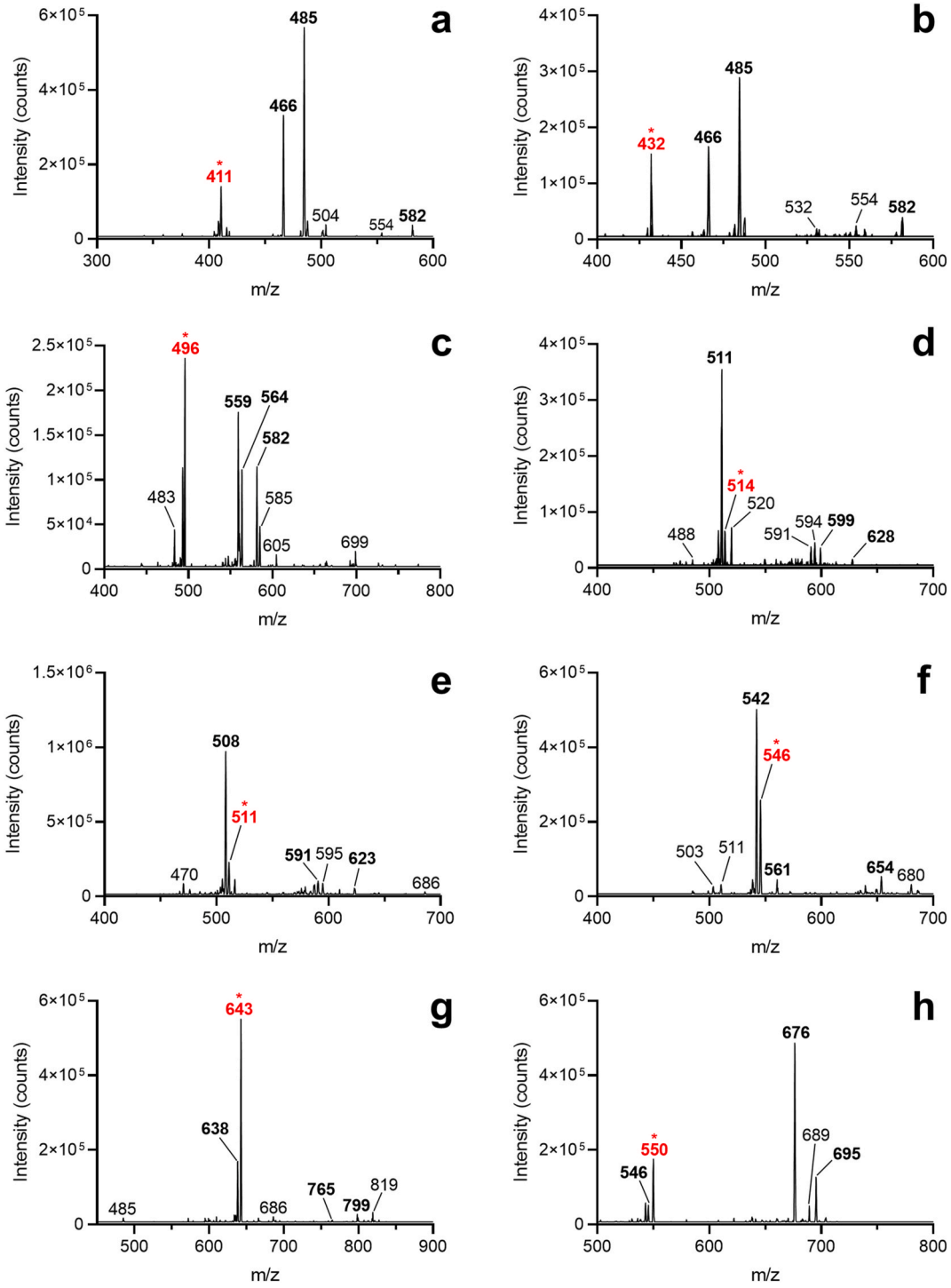

Fig. 1. Product ion mass spectrum of a standard solution of a) BNP 3–32, b) BNP 5–32, c) BNP 1–32, d) ANP 1–28, e) ANP (rat), f) ANP 4–28, g) ANP 5–28, h) CNP at a concentration of 1 μg/mL and acquired at the collision energy of the quantitative transition. Precursor ions are labeled in bold red and selected product ions in bold black.

guarantee at least 18–20 points for each chromatographic peak. The mass resolution (full width at half maximum; FWHM) was set at 1.2 Da for both quadrupoles. Fig. 1 shows the product ion mass spectra acquired at a concentration of 1 μg/mL for all the target analytes at the quantifier transition collision energy.

Molecular weight, retention time (RT), charge state of the selected precursor ion, MRM quantifier (Q), and qualifier (q) transitions with corresponding collision energy (CE), and qualifier-to-quantifier ratio are reported in Table 1.

Peptides intermediate stock solutions (1 μg/mL) were also analyzed in flow injection mode using a 1200 Infinity HPLC coupled to a 6530 Accurate-Mass Quadrupole Time-Of-Flight (Q-ToF) mass spectrometer equipped with a Jet Stream ESI interface (Agilent Technologies, USA) to acquire high-resolution mass spectra. ToF mass resolution (FWHM) was 10,000 at m/z 118 and 20,000 at m/z 1522, whereas quadrupole resolution was set at 1.3 Da. The autosampler temperature was kept at room temperature (25 °C). Source parameters were the same used with the triple quadrupole mass spectrometer, except for the fragmentor (150 V). The collision energy (CID) for the MS/MS experiments was the same as reported in Table 1.

Both instruments were controlled by MassHunter™ software (Agilent Technologies, USA).

Table 1
Molecular weight, retention time, charge state of the selected precursor ion, quantifier and qualifier MRM transitions with corresponding collision energy (CE), and qualifier-to-quantifier ratio for all the investigated compounds.

Compound	MW (Da)	RT (min)	Charge	MRM parameters			
					Transitions (*m/z*)	CE (eV)	q/Q ratio
BNP 3-32	3282	4.59	$[M+8H]^{8+}$	**Q**	411 → 485	8	–
				q1	411 → 466	8	0.79
				q2	411 → 582	8	0.04
BNP 5-32	3023	4.69	$[M+7H]^{7+}$	**Q**	432 → 485	14	–
				q1	432 → 466	14	0.63
				q2	432 → 582	13	0.10
BNP 1-32	3464	5.01	$[M+7H]^{7+}$	**Q**	496 → 559	14	–
				q1	496 → 564	14	0.74
				q2	496 → 582	15	0.61
ANP 1-28	3081	7.24	$[M+6H]^{6+}$	**Q**	514 → 511	8	–
				q1	514 → 599	11	0.06
				q2	514 → 628	11	0.06
ANP (rat)	3063	7.55	$[M+6H]^{6+}$	**Q**	511 → 508	10	–
				q1	511 → 591	10	0.05
				q2	511 → 623	10	0.04
ANP 4-28	2722	7.56	$[M+5H]^{5+}$	**Q**	546 → 542	10	–
				q1	546 → 561	11	0.12
				q2	546 → 654	11	0.11
ANP 5-28	2568	8.26	$[M+4H]^{4+}$	**Q**	643 → 638	18	–
				q1	643 → 799	20	0.31
				q2	643 → 765	22	0.06
CNP	2198	10.47	$[M+4H]^{4+}$	**Q**	550 → 676	13	–
				q1	550 → 546	14	0.22
				q2	550 → 695	13	0.17

2.4. Preparation of standard mixtures and quality control samples

Stock solutions were prepared by reconstituting each lyophilized analyte with 1000 μL of a H_2O:ACN mixture (90:10 % v/v) containing formic acid (0.2 % v/v). Each solution was stirred at 3000 rpm until the peptide completely dissolved. Intermediate stock solutions of each analyte and IS were prepared at a concentration of 1 or 5 (for peptides) and 1 μg/mL (for IS) by diluting appropriate aliquots of stock solutions with a H_2O:ACN mixture (90:10 % v/v) containing formic acid (0.2 % v/v). Stock and intermediate solutions were kept in sterile LoBind Eppendorf tubes and stored at −80 °C until their use.

A stock solution of methionine (3 mM) was prepared by dissolving 4.5 mg of the pure compound into 10 mL of water and then stored at 4 °C for up to two months according to the manufacturer's instructions.

An aqueous solution was prepared by dissolving 5 mg of HSA with 10 mL of water containing methionine (0.3 mM) and formic acid (0.2 % v/v). The resulting solution was then stored at −80 °C for up to a week to prevent degradation of HSA and methionine. This solution was used to prepare working solutions of all target peptides at a concentration level of 2.5, 5, 10, 20, and 50 ng/mL. All these solutions were stored at −80 °C in sterile LoBind Eppendorf tubes and used for up to a maximum of 5 freeze-thaw cycles and for up to 1 month.

An aqueous solution, namely reconstituting solution, was prepared by diluting the methionine stock solution (3 mM) ten times with water containing trifluoroacetic acid (0.5 % v/v).

Plasma samples were collected at Santa Chiara Hospital thanks to the collaboration with Dr. Nicola Pugliese and his team. A pooled human plasma was obtained by mixing aliquots (1000 μL) of plasma collected from 50 patients suffering from HF and/or hypertension. All samples were immediately stored at −80 °C in 50 mL Falcon tubes and were then shipped to the laboratory for analysis. After the thawing process at room temperature (25 °C), samples were stirred at 2500 rpm for several min to ensure homogenization, separated into aliquots of 1.5 mL each, and then stored at −80 °C in sterile LoBind polypropylene tubes. Each aliquot was subjected to 1 freeze-thaw cycle. Aliquots of human pooled plasma samples were spiked with known amounts of NPs to obtain fresh quality control samples at three concentration levels: 100, 200, and 500 pg/mL.

2.5. Plasma sample preparation

2.5.1. Procedure A: ultrafiltration with a 10 kDa membrane cut-off

Preliminary tests were performed by centrifuging an aliquot (500 μL) of a standard solution, which contained all target peptides at a concentration of 10 ng/mL, for 1 h at 19496×*g* and 4 °C using an Amicon® Ultra 10 kDa device. The retentate was recovered by turning the filter upside down and centrifuging it at 25 °C for 2 min at 1579×*g*, as recommended by the device specifications. The regenerated cellulose membrane and the collection tube of the centrifugal device were treated with an aliquot (500 μL) of a MeOH:H_2O (50:50 % v/v) mixture to extract any peptides that may have been potentially adsorbed on the filter or retained by the plastic tube. The amount of methanol in the extraction mixture was selected based on the maximum percentage of organic solvent that the centrifugal device can tolerate. Finally, all samples (i.e., filtrate, retentate, analytes adsorbed on the filter, analytes adsorbed to tube surfaces) were analyzed by UHPLC-MS/MS.

An aliquot of a quality control plasma sample (500 μL) at a concentration of 0.5 ng/mL for each peptide was spiked with 20 μL of P-8340 protease inhibitor cocktail and centrifuged for 1 h at 19496×*g* and 25 °C using an Amicon® Ultra 10 kDa device. The ultrafiltrate (300 μL) was then transferred into a 5 mL sterile protein LoBind polypropylene

tube, diluted to 3000 μL with the reconstituting solution containing TFA (0.5 % v/v) and methionine (0.3 mM) and then subjected to MEPS (see procedure B for MEPS parameters).

2.5.2. Procedure B: salting-out of proteins coupled with microextraction by packed sorbent

An aliquot (500 μL) of both quality control (0.5 ng/mL of each peptide) and blank samples was spiked with 20 μL of P-8340 protease inhibitor cocktail and with 8 μL of ANP (rat) (30 ng/mL). The mixture was then combined with a saturated solution of ammonium sulfate (4500 μL) and stirred for 2 min at 3000 rpm. After 10 min of centrifugation at 19496×*g* and room temperature (25 °C), the supernatant was discarded. The remaining protein pellets were treated with an aliquot (1000 μL) of MeOH containing TFA (0.5 % v/v) and stirred for 1 min at 2500 rpm. The sample was heated at 50 °C for 30 min and stirred at 1000 rpm in a Thermo Mixer device. Then, the sample was further centrifuged for 10 min at 19496×*g* and 25 °C. The supernatant was filtered by a Phenex™-RC syringe filter (0.2 μm) and the volume was reduced to 200 μL under a gentle stream of nitrogen. The residue sample was diluted with 3000 μL of an aqueous solution (reconstituting solution) containing TFA (0.5 % v/v) and methionine (0.3 mM) and stirred for 1 min at 2500 rpm. To ensure the complete solubilization of all the target peptides, the solution was heated at 50 °C for 5 min at a rate of 1000 rpm in the Thermo Mixer device. Finally, the solution was processed with the MEPS procedure for a further clean-up step.

The MEPS cartridge (BIN C18) was activated by drawing and discharging 3 times 100 μL of methanol (3 × 100 μL) and then conditioned with 3 cycles of drawing and discharge of 100 μL each of a water solution containing TFA (0.5 % v/v). The diluted residue from the salting-out step (3000 μL) was loaded up and down ten times in discharge mode, allowing the target peptides to be retained by the C18 sorbent. The cartridge was then washed with 100 μL of an aqueous solution containing methionine (0.3 mM) and TFA (0.5 % v/v) to remove potential interferents and avoid analyte oxidation. Analytes were eluted with 2 aliquots of 35 μL of H_2O:MeOH (20:80 v/v) containing formic acid (0.1 % v/v). The extracts were injected into the UHPLC-MS/MS instrument for the final analysis. At the end of the MEPS procedure, BIN was washed 2 times with 250 μL of H_2O:MeOH (10:90 v/v) containing formic acid (0.1 % v/v). All steps were performed at a flow rate of 0.18 mL/min.

Fig. 2 shows the overall steps of the final analytical workflow developed in this work.

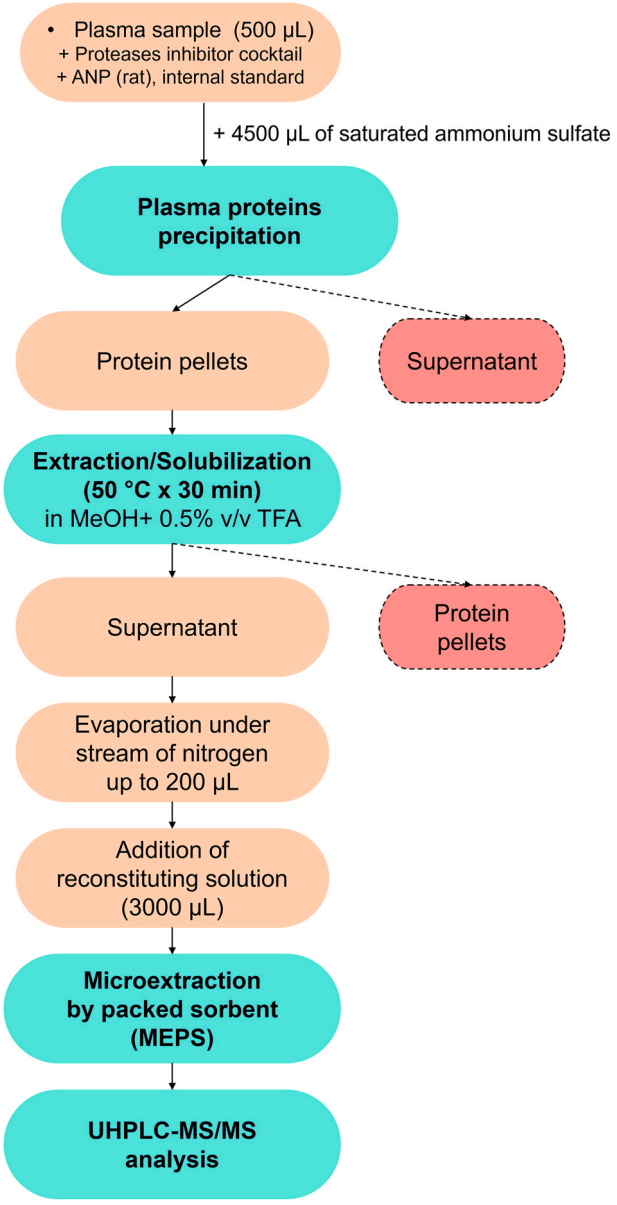

Fig. 2. A complete overview of the analytical workflow for the determination of natriuretic peptides in human plasma. Light red boxes with dotted lines represent part of the sample discarded during the procedure. Light green boxes with bold characters denote the principal steps of the procedure. TFA: trifluoroacetic acid.

2.6. Method validation

The analytical protocol was validated following the guidelines of the International Union of Pure and Applied Chemistry (IUPAC) [26]. Quality control samples containing ANP 1–28, ANP 5–28, BNP 5–32, and CNP at a concentration of 25, 50, 100, 200, and 500 pg/mL, and ANP 4–28, BNP 1–32, and BNP 3–32 at concentration of 50, 100, 200, and 500 pg/mL were spiked with 20 μL of P-8340 protease inhibitor cocktail and 8 μL of IS (30 ng/mL) to prepare internal calibration solutions.

Each calibration level was analyzed using procedure B in triplicates on a single day and on three consecutive days to estimate the intra- and inter-day precision, respectively. The recovery was calculated as the percentile ratio of the difference between the measured analyte concentration in the spiked and unspiked samples (if the signal was present in the blank) to the nominal spiked concentration. The analyte (a) to internal standard (IS) peak area ratios (Y, Aa/AIS) were plotted against the corresponding concentration ratios (X, Ca/CIS). The calibration curves ($Y = mX$) for all peptides were evaluated using Deming regression analysis, which considers measurement errors for both dependent and independent variables [27].

Limits of detection (LOD) and quantification (LOQ) were calculated as the ratio between three and ten times the standard deviation of the lowest spiked sample, and the slope of the calibration curves for all the investigated compounds, respectively.

The presence of matrix effect was evaluated by comparing the slope between calibration curves carried out by spiking standard solutions of NPs at different concentrations into pooled plasma samples with that obtained by analyzing working standard solutions at different concentration levels.

2.7. Stability study

The occurrence of oxidized species of each peptide was evaluated at room temperature (25 °C) by exposing three independent open vials, each containing a standard working solution (1 μg/mL), to atmospheric conditions. Analysis was performed at t_0 and after 24, 36, and 72 h of exposure. Peptide peak areas at t_0 were used as reference values.

The stability of each peptide in standard working solutions prepared at a concentration level of 2 ng/mL in a H_2O:ACN mixture (90:10 % v/v)

containing formic acid (0.2 % v/v) was evaluated at 4 and 25 °C for up to 24 h. The addition of HSA (0.05 % w/v), methionine (0.3 mM), and BHT (15 mg/mL) into the dilution solvent was tested to prevent peptide oxidation [28] and/or adsorption on the vial walls [25].

To simulate a routine application of the entire analytical workflow during a typical working day, the stability of the target peptides was evaluated at 25 °C and 4 °C by analyzing both the reconstituting solution (after salting-out precipitation, prior to the MEPS procedure) and the MEPS extract. In particular, the first solution was analyzed at t_0 and after 3, 6, and 15 h from the preparation to estimate the short-term stability at 25 °C, as well as after 1, 3, and 7 days for the mid-term stability at 4 °C. Similarly, MEPS extracts were analyzed at t_0 and after 3, 6, and 24 h to evaluate the short-term stability at both 25 and 4 °C.

In all experiments, the peak area of each analyte measured at t_0 was used as the reference value. ANOVA at a confidence level of 95 % was used to compare the stability results. Each experiment was performed in triplicate.

2.8. Effect of collection procedure on natriuretic peptides levels in blood and preliminary data on heart failure patients

The degradation of biologically active natriuretic peptides (i.e., ANP 1–28, BNP 1–32, and CNP) in plasma samples at room temperature was evaluated. This was done by spiking 20 μL of P-8340 protease inhibitor cocktail and 8 μL of ANP (rat) (30 ng/mL) into an aliquot (500 μL) of quality control plasma sample (0.5 ng/mL of each peptide, n = 2). Analyses were performed just right after the preparation (t_0) and 5 and 20 min after adding the exogenous peptides to the plasma. The chosen times were based on half-life data reported elsewhere [29].

The effect of sampling on NPs levels in blood was tested by collecting three blood samples from four HF patients enrolled at Santa Chiara Hospital (Pisa). BD™P100 spray-coated test tubes containing peptide stabilizers and protease inhibitors were used. Plasma samples were obtained by centrifuging (2500×*g* according to the manufacturer) test tubes at t_0, i.e., immediately after drawing the blood, and then after 1 and 2 h from the collection, while keeping the tubes at 4 °C. All plasma samples were spiked with 20 μL of P-8340 protease inhibitor cocktail and then stored at −80 °C until use.

The validated analytical platform was used to monitor 13 consecutive patients with a prior clinical diagnosis of heart failure during a routine follow-up visit. The protocol was approved by the local ethics committees (number 19204) in accordance with the 1964 Declaration of Helsinki and subsequent updates. Written informed consent was obtained from all patients. The population consisted of 7 males and 6 females, with an average age of 79 ± 7 years, a minimum age of 70 years, and a maximum age of 89 years. Plasma NT-proBNP levels were obtained using the hospital procedure based on a commercial automated electrochemiluminescent immunoassay (Elecsys NT-proBNP II, Roche Diagnostics).

Data were analyzed using GraphPad Prism (v. 8.0) from GraphPad Software Inc. (La Jolla, USA) and R software (RStudio, USA).

3. Results and discussion

3.1. Optimization of UHPLC-ESI-MS/MS method

The optimization of the chromatographic separation was performed by systematically testing three RRHD Zorbax columns (300-SB C3, 300-SB C18, and SB-Aq C18), methanol and acetonitrile as organic modifiers, and different amounts (0.1, 0.2, and 0.3 % v/v) of formic acid as an additive of the mobile phase. Additionally, the effect of column flow rate (0.2–0.5 mL/min) and temperature (25, 40, 50, 60, and 70 °C) was also evaluated. Higher column temperatures were excluded to avoid cleavage of the intramolecular disulfide bond [30].

The selected columns have the same specifications (2.1 × 100 mm, 1.8 μm particle size), but they differ in pore size (300 Å for both 300-SB C3 and C18 columns, and 80 Å for SB-Aq C18 column) and bonded alkyl chain (C3 *vs.* C18). The use of the SB-Aq column would ensure better interaction with the sorbent phase in the case of polar analytes (such as BNPs) due to the phase's stability at high water percentages, according to the manufacturer. However, the chromatographic separation with the SB-Aq column was not satisfactory due to a clear coelution of BNPs that eluted just after the column dead volume. In comparison, both 300-SB columns exhibited better chromatographic behavior as different percentages of organic solvents led to modification of the retention time and peak shape. This could be attributed to the larger pore size of the stationary phase, as discussed elsewhere [31]. The use of a 300-SB C18 column ensured strong retention of the analytes even at a high percentage of organic solvent, resulting in increased run time and peak tailing. As a general observation, methanol increased analyte retention, while acetonitrile led to shorter retention times and narrower peaks, especially for the more polar peptides. For example, the full width at half maximum (FWHM) of BNP 1–32 was 0.36 and 0.10 min with methanol and acetonitrile, respectively. Thus, the best compromise was achieved using a mobile phase composed of water and acetonitrile. The highest peak intensity for all analytes was obtained by adding formic acid (0.2 % v/v) to both mobile phase components. Under these conditions, the signal enhancement was approximately 40 %. Increasing the column temperature resulted in better chromatographic separation among BNP 1–32 and its homologous forms. For example, the separation factor (α) at 25 °C between BNP 1–32 and BNP 3–32 was 1.04. However, 50 °C was selected as the optimal temperature since a reduction in peak signal was observed at 60 and 70 °C. Flow rates lower than 0.4 mL/min caused overlap of the BNP 3–32, BNP 5–32, and BNP 1–32 peaks, while a flow rate of 0.5 mL/min resulted in high back pressure in the LC system. Therefore, a flow rate of 0.4 mL/min was chosen to achieve chromatographic separation.

The addition of supercharger reagents, such as 3-nitrobenzyl alcohol (3-NBA), dimethyl sulfoxide (DMSO), or glycerol, to the mobile phase, has been proposed to enhance the electrospray ionization process of large molecules [32]. The addition of superchargers to mobile phases may cause a shift to higher charge state distributions of protein mass spectra [33]. Fig. S2 shows a representative example of the shifts in the charge state distribution of BNP 1–32. As expected, the addition of a supercharger reagent lowered the *m/z* values of the mass spectrum, leading to higher charge states [32]. Our results highlighted a superior effect of 3-NBA compared to DMSO, as a general increase (at least two times) in the signal-to-noise ratio was observed for all the target peptides, confirming what was observed for BNP 1–32 by Torma et al. [22]. Table S1 reports the increases in the S/N ratio observed with DMSO and 3-NBA.

To summarize, the best achieved chromatographic conditions were obtained using a Zorbax 300-SB C3 column at 50 °C, using a mobile phase composed of formic acid (0.2 % v/v) and 3-NBA (0.01 % v/v) aqueous solution (A), and ACN containing formic acid (0.2 % v/v) and 3-NBA (0.01 % v/v) (B) at a flow rate of 0.4 mL/min.

Fig. 4a shows a typical MRM chromatogram of a standard solution containing all target peptides at a concentration of 500 pg/mL. To the best of our knowledge, this is the first LC-MS method allowing a satisfactory separation of most of the native NPs and some of their degraded forms (i.e., BNP 3–32, BNP 5–32, ANP 4–28, and ANP 5–28).

High-resolution mass spectrometry confirmed the charge state of the peptide precursor ions and helped to identify some fragments of the molecules, as shown in Fig. S3 for CNP (*m/z* = 550.281). The fragment ions identified were the quantifier ion of CNP (676.669 *m/z*), which is generated by the loss of the dipeptide Gly-Leu at the N-terminal part (with a contemporary loss of a positive charge), whereas the qualifier ion 546.022 *m/z* corresponds to the loss of a water molecule. Fragment ions of BNP 1–32, BNP 3–32, and BNP 5–32 were successfully identified. In the case of BNP 1–32, we confirmed the loss of serine from the N-terminal side of the peptide with the contemporary loss of a positive charge (*m/z* 563.617). Moreover, the quantifier ions of ANP 1–28 at

511.407 *m/z*, ANP 4–28 at 542.348 *m/z*, ANP 5–28 at 638.625 *m/z*, and ANP (rat) at 508.252 *m/z* were all attributable to the loss of a water molecule with no loss of positive charges from the peptide.

3.2. Development of a reliable plasma preparation procedure

3.2.1. Procedure A: ultrafiltration with a 10 kDa membrane cut-off

Fig. 3 shows the distribution of the analytes within the Amicon device, i.e. ultrafiltrate, retentate, plastic walls of the tube, and filter membrane, after centrifugation of a standard solution of NPs at a concentration level of 10 ng/mL.

ANPs were distributed almost equally among the different phases, with recoveries ranging from 25 to 40 %. This suggests limited membrane permeation for these peptides, but also a significant interaction with the plastic material that makes up the centrifugal device. BNPs showed limited adsorption on both the plastic walls and the filter but had a similar distribution between the ultrafiltrate and the retentate. On the other hand, CNP was mainly adsorbed on the filter (about 75 %), with a small amount of peptide found in the ultrafiltrate, even though its molecular weight (2198 Da) is lower than the membrane cut-off. Since the presence of matrix may decrease the non-specific adsorption of the analytes to plastic surfaces, the ultrafiltration procedure was also tested using a spiked pooled plasma sample (n = 2), containing both the target analytes and IS at a concentration of 500 pg/mL. The analysis was immediately stopped because of the production of large air bubbles within the MEPS syringe highlighting the need for an ulterior pre-treatment step after the ultrafiltration. The presence of non-specific interactions of NPs within the ultrafiltration device and the need for an ulterior pre-treatment step led to the abandon of procedure A.

3.2.2. Procedure B: salting-out of proteins coupled with MEPS

Salting-out has been tested by applying one or more subsequent additions of saturated ammonium sulfate (i.e., fractional protein precipitation) [16]. Herein we tested the addition of ammonium sulfate up to 20 and 35 % v/v followed by a second addition reaching a final salt content of 50, 70, or 90 % v/v. Results suggested an incomplete protein precipitation at 20 % of saturated ammonium sulfate whereas the addition of up to 35 % leads to a partial precipitation of the target peptides Thus, considering the difficulties in defining a suitable percentage of ammonium sulfate, we tested a different approach based on the quantitative precipitation of both target analytes and matrix proteins, followed by resuspension with an appropriate solvent. Tests with various amounts of ammonium sulfate (50, 70, and 90 %) highlighted an almost quantitative NPs precipitation at 90 % (Fig. S4). Solubilization experiments using pure methanol or in combination with water (MeOH: H_2O mixtures, 90:10, 80:20, and 70:30 % v/v), with and without TFA as an ion pair reagent, were performed. The best results were achieved using 1000 μL of MeOH containing TFA (0.5 % v/v). Tests were then

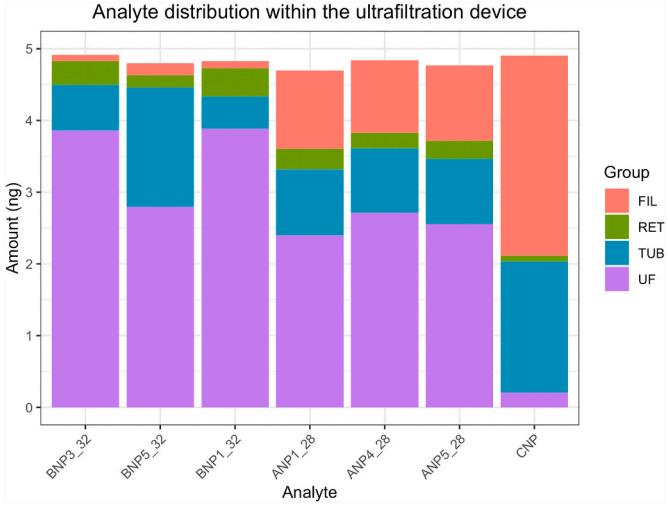

Fig. 3. Distribution of target analytes in the ultrafiltrate (UF, purple), in the retentate (RET, green), adsorbed in the plastic walls (TUB, light blue), and adsorbed in the filter (FIL, red).

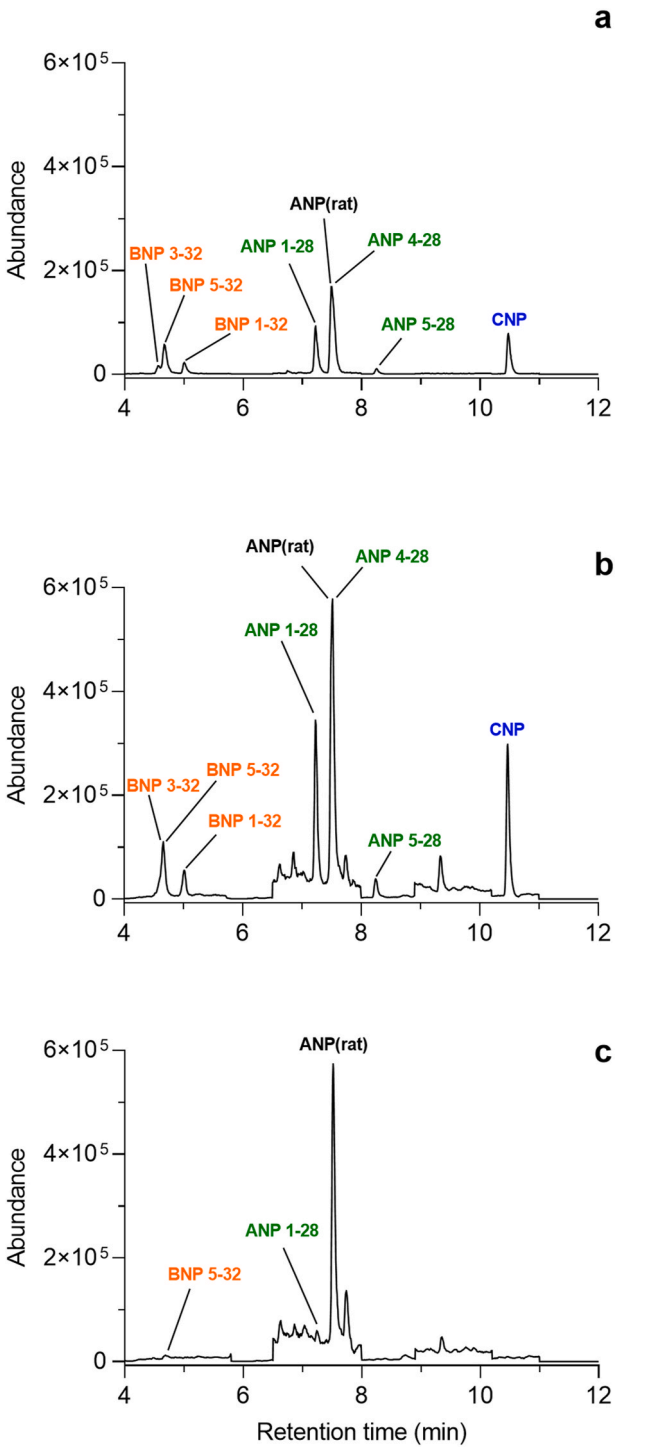

Fig. 4. TIC MRM chromatogram of (a) standard solution containing ANP 1–28, ANP 4–28, ANP 5–28, ANP (rat), BNP 1–32, BNP 3–32, BNP 5–32, and CNP (500 pg/mL of each peptide), (b) pooled blank plasma sample spiked with target analytes and IS (both at 500 pg/mL), and (c) pooled blank plasma sample spiked with IS (500 pg/mL). Both plasma samples were subjected to salting-out with ammonium sulfate followed by MEPS procedure. IS: internal standard, ANP (rat).

extended to evaluate the effect of temperature (25, 40, 50, and 60 °C), time (0, 5, 15, and 30 min), and mixing speed (500 and 1000 rpm). Results showed that the best conditions to maximize the extraction yield for all the analytes were: 50 °C, 30 min, and 1000 rpm. After solubilization, the organic supernatant was filtered at 0.2 μm with a syringe filter into a low-bind tube, and then evaporated under a gentle stream of nitrogen up to 200 μL. The resulting supernatant was diluted up to 3000 μL with the reconstituting solution (aqueous solution of methionine (0.3 mM) and TFA (0.5 % v/v), to prevent oxidation of the peptides during MEPS clean-up and increase the interactions between peptides and sorbent material. No significant analyte losses were observed during the filtration.

Regarding MEPS clean-up, C18 and C2 stationary phases were tested to select a reliable sorbent. Compared to C2 sorbent, C18 significantly enhanced (up to 3-fold) the analyte retention leading to higher recovery values, especially for the more polar analytes (e.g., BNP 1–32 36 % for C2 and 98 % for C18). The washing procedure was carried out using a methionine aqueous solution (0.3 μM) containing TFA (0.5 % v/v) preventing the oxidation of BNP 1–32 during the clean-up [22]. A partial elution (70 %) from the sorbent material was observed performing a single elution with 50 μL of MeOH:H_2O mixture (80:20 % v/v) containing formic acid (0.1 % v/v). A cyclic elution with two aliquots (35 μL each) of the same mixture increased the extraction up to about 90 %. Notably, more than 40 samples were loaded in a row on the same BIN without affecting extraction performance.

Fig. 4 shows the total ion chromatogram acquired in MRM mode of a standard solution containing target peptides and IS (both at 500 pg/mL), a pooled blank plasma sample spiked with the target peptides and IS (both at 500 pg/mL), and a pooled blank plasma sample spiked with IS (500 pg/mL). Both plasma samples were subjected to salting-out with ammonium sulfate followed by MEPS procedure, whereas the standard solution was analyzed without any further manipulation.

3.3. Stability study

3.3.1. Stability of peptides standard solutions

Fig. S5 shows a representative example of the trend of the BNP 1–32 signal over time obtained by analyzing a standard working solution (2 ng/mL) prepared in 90:10 % v/v H_2O:ACN mixture containing formic acid (0.2 % v/v) and kept in a polypropylene vial. After 4 h of storage at 4 °C, a marked decrease (−91 %) of the signal was observed for all the analytes. This decrease is likely due to a combination of factors, such as a non-specific binding process between the analytes and plastic, as well as poor analyte stability under atmospheric conditions. Interestingly, standard solutions with a higher concentration (≥1 μg/mL) of peptide did not exhibit the same behavior. This difference is probably due to a less non-specific binding process in the presence of a higher concentration of peptides in the solution [34]. Fig. 5a shows the presence of additional peaks (labeled in pink and blue) in the MS2Scan chromatogram obtained from the oxidation process of BNP 1–32 after 24 h of ambient exposure. Considering the lower retention time of the oxidative forms, we postulated a decrease in the peptide's hydrophobicity due to the oxidation of methionine(s) [35]. The presence of three additional peaks in the chromatogram confirmed the production of both oxidative forms. Interestingly, a clear increasing trend over time (up to 72 h) was observed for the oxidative form with two oxidized methionines, whereas the other forms (with one oxidized methionine) showed an increase of 20–30 % after 24 h, followed by a decrease at later times, suggesting further oxidation of the second methionine residue.

BNP 5–32 and ANPs have a single methionine within their structures, so oxidation produces one additional peak that is clearly visible in the MS2Scan chromatogram (Fig. S6). In this case, as well, a clear increasing trend over time was observed. CNP appears to have a greater tendency to oxidation compared to other NPs, as the oxidized form is already the most abundant after 24 h of ambient exposure (Fig. S6). Therefore, based on these results, we tested the addition of BHT (15 mg/mL) and methionine (0.3 mM) as oxygen scavengers to prevent peptide oxidation, and HSA (0.05 % w/v) to minimize their non-specific adsorption on the polypropylene vial walls. The results showed a significant difference between BHT and methionine, with only methionine improving the stability of peptides, although a significant decrease in signal (about 30 %) was still observed after 24 h of storage. The addition of HSA further stabilized the peptide signals, as no significant variations were observed after 16 h. This could be due to the scavenger effect of HSA, which contains an appreciable amount of methionine group within its structure, as well as its capability to minimize the adsorption of peptides on vial walls. Similar results were also obtained at room temperature for up to 24 h.

3.3.2. Stability of analytes in reconstituting solution and MEPS eluate

The results showed that all the analytes were stable in the reconstituting solution, methionine aqueous solution (0.3 mM) containing TFA (0.5 % v/v), for at least 6 h at 25 °C and up to 7 days at 4 °C. Only BNP 1–32 showed a significant decrease after seven days of storage at 4 °C, likely due to partial oxidation at methionine residues. The occurrence of non-specific binding was ruled out by using specific low binding containers. Regarding the stability of the MEPS eluate solutions, all analytes remained stable under the tested conditions, suggesting that the elution mixture (MeOH:H_2O 80:20 % v/v containing 0.1 % v/v of FA) prevents secondary interactions or peptide modification over time. These findings support the potential of automating the MEPS procedure and increasing laboratory throughput. For example, our automatic MEPS system can process up to 18 samples in the same sequence.

3.4. Analytical figures of merit.

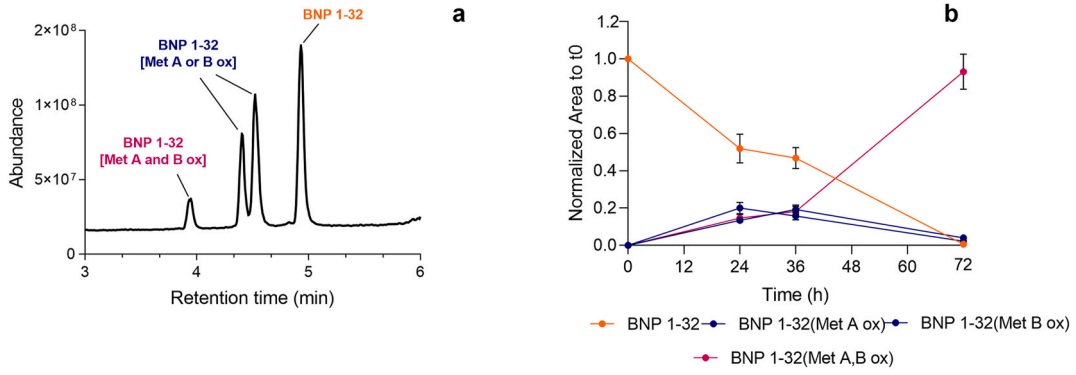

Fig. 5. (a) MS2Scan chromatogram of a standard working solution (1 μg/mL) of BNP 1–32 prepared in 90:10 % v/v H_2O:ACN mixture containing formic acid (0.2 % v/v) after 24 h of exposure to ambient conditions; (b) Trend over time of BNP 1–32 and its oxidative forms. Peak areas were normalized to the corresponding peak area observed at t0. The labels indicate the different forms of BNP 1–32, with native BNP 1–32 in orange, BNP 1–32 with one oxidized methionine (A or B) in blue, and BNP 1–32 with two oxidized methionine (both A and B) in pink.

Table 2 reports, for all the investigated analytes, the internal and external calibration levels, slope (average and standard deviation), and limits of detection and quantification, as well as the intra- and inter-day recovery with their corresponding relative standard deviation (RSD).

The chromatographic signal intensity exhibited good linearity ($R^2 \geq 0.99$) within the investigated concentration ranges for all the target compounds. The matrix effect was assessed by performing a *t*-test at a confidence level of 95 % between the slopes of the two calibration curves. The slopes were found to be significantly different at a confidence level of 95 %, indicating the presence of a matrix effect. Since the presence of matrix effect may affect measurements, we performed the quantification of NPs by using an internal calibration with spiked pooled plasma samples at different concentrations. The recovery of all the analytes fell within the range of 94–105 %, while the intra- and inter-day precisions were below 20 %. The LOD values ranged from 0.2 to 20 pg/mL confirming the reliability of the analytical protocol to quantify natriuretic peptides in plasma samples. These values were below the threshold of NPs concentration reported in clinical heart failure guidelines [3].

3.4. Evaluation of blood sample collection for the analysis of natriuretic peptides: preliminary data from heart failure patients

Significant changes (20–25 %) in the plasma concentration of BNP 1–32 were observed after 5 min of storage at room temperature. These changes reached a decrease of up to 40–50 % after 20 min, confirming findings in the literature regarding the presence of plasmatic proteases, such as neprilysin (NEP) and dipeptidyl peptidase IV (DPP IV), which can degrade BNP 1–32 into several biologically inactive analogs [23]. Only a limited number of studies have examined the stability of ANP 1–28 in human plasma [36–38]. These studies highlighted the poor stability of ANP under storage conditions at −80 °C [37] and at room temperature [36] in the absence of a proper protease inhibitor. However, others have indicated the stability of ANP under storage conditions at −80 °C [38]. Our data confirmed the stability of ANP 1–28 in plasma at room temperature, as no statistically significant changes ($p > 0.05$) were observed over time. In contrast, CNP exhibited a significant ($p < 0.05$) decrease of 20 % after a 20 min storage, which disagrees with other studies [39]. However, it should be noted that the lack of standardization and harmonization of NPs immunoassays may lead to confounding results in the stability studies.

Our data suggest that common blood collection tubes containing EDTA are not suitable to quantify accurately peptides in plasma samples [21]. Thus, we tested BD™P100 spray-coated tubes containing peptide stabilizers and protease inhibitors as alternative devices for blood collection [40]. No significant changes ($p > 0.05$) in the analyte concentration were observed within 2 h of blood storage at room temperature, confirming that particular care is needed to preserve samples during the pre-analytical stage of the procedure.

Table 3 reports the concentration of NPs measured in plasma samples collected using BD™ P100 tubes from a small group of HF patients enrolled in the study to test the reliability of the method. ANP 4–28 and CNP concentrations were below the limit of detection in all the samples, whereas ANP 5–28 was quantified in only 2 out of 13 samples. To the best of our knowledge, we detected ANP 5–28 in human plasma by mass spectrometry for the first time, although it is unclear whether it is involved in human biological processes or not. Contrary, ANP 1–28 was observed in all plasma samples, with concentrations ranging from 25 to 543 pg/mL. This peptide is known to be released from cardiomyocytes in response to myocardial stretch to regulate blood pressure. In our data, BNP 1–32 was observed in just one sample at a concentration of 112 pg/mL, whereas BNP 5–32 was found to be the major circulating form of BNPs. Literature suggests that this molecule to be is one of the major circulating forms of BNPs involved in HF pathophysiological processes [10]. Interestingly, patient P13 had NT-proBNP levels slightly higher than the acute HF cut-off (<1800 pg/mL, age >75 [41]) but showed high levels of NPs. The high concentration of BNP 5–32 (1005 pg/mL) may be due to its production in vivo through the degradation of BNP 1–32 operated by NEP [10]. Some studies have already reported the utility of BNP 5–32 as a biomarker of HF prognosis in combination with NT-proBNP [42].

We believe that our work, focused on the quantification of biologically active NPs (and of some degradation products), may help elucidating the role of these peptides in the pathophysiology of HF. Particularly, the analysis of an array of NPs could aid physicians in diagnosing and assessing the prognosis of HF, paving the way to the personalization of therapies.

4. Conclusions

In this paper, we present the first UHPLC-ESI-MS/MS platform for determining a panel of intact natriuretic peptides (i.e., ANP 1–28, ANP 4–28, ANP 5–28, BNP 1–32, BNP 3–32, BNP 5–32, and CNP) in human plasma samples. The stability study on NPs aqueous standard solution highlighted their tendency to adsorb on polypropylene HPLC vial surfaces and/or to be oxidized at methionine residues when exposed to ambient air. Our data suggest that the use of an aqueous mixture of methionine 0.3 mM, human serum albumin 0.05 % w/v, and formic acid 0.2 % v/v avoid these phenomena for 24 h.

The analytical platform combines the salting-out of plasma proteins with saturated ammonium sulfate, followed by an automated MEPS procedure for cleaning up the sample and pre-concentrating the analytes prior to analysis. The performances in terms of precision, recovery, linearity, and sensitivity allowed us to achieve limits of detection below the cut-off in clinical HF guidelines, demonstrating the suitability of the procedure for monitoring HF patients.

Preliminary results show the importance of using blood collection tubes containing protease inhibitors to avoid peptide degradation during sample storage and manipulation. Data obtained from the analyses performed on a small set of plasma samples show the presence of ANP 1–28 as the most abundant analyte, followed by BNP 5–32, ANP 5–28, and BNP 1–32.

Table 2
Calibration levels and slopes of external and internal calibration curves (average and standard deviation), limits of detection (LODs) and quantification (LOQs), intra-day and inter-day recovery for the determination of NPs in plasma samples.

Compound	Calibration levels (pg/mL)	External calibration (slope±s.d.)	Internal calibration (slope ± s.d.)	LOD (pg/mL)	LOQ (pg/mL)	Intra-day recovery % (RSD*)	Inter-day recovery % (RSD*)
ANP 1–28	25; 50; 100; 200; 500	0.58 ± 0.05	0.52 ± 0.02	0.2	0.7	99 (10)	96 (9)
ANP 4–28	50; 100; 200; 500	0.45 ± 0.04	0.33 ± 0.02	11	37	99 (8)	100 (10)
ANP 5–28	50; 100; 200; 500	0.10 ± 0.01	0.070 ± 0.006	18	60	105 (12)	101 (14)
BNP 1–32	50; 100; 200; 500	0.082 ± 0.008	0.0533 ± 0.0001	20	67	100 (10)	99 (7)
BNP 3–32	50; 100; 200; 500	0.068 ± 0.007	0.037 ± 0.001	15	50	97 (19)	94 (18)
BNP 5–32	25; 50; 100; 200; 500	0.28 ± 0.03	0.18 ± 0.02	9	30	100 (12)	97 (8)
CNP	25; 50; 100; 200; 500	0.52 ± 0.05	0.46 ± 0.03	5	17	99 (10)	96 (11)

[a]RSD: relative standard deviation.

Table 3
Concentration of target natriuretic peptides measured in blood collected from heart failure patients.

Patient ID	NT-proBNP (pg/mL)[a]	BNP 3–32 (pg/mL)	BNP 5–32 (pg/mL)	BNP 1–32 (pg/mL)	ANP 1–28 (pg/mL)	ANP 4–28 (pg/mL)	ANP 5–28 (pg/mL)	CNP (pg/mL)
P1	24488	<LOD	40	<LOQ	127	<LOD	<LOQ	<LOD
P2	556	<LOD	<LOQ	<LOD	70	<LOD	<LOQ	<LOD
P3	980	<LOD	<LOQ	<LOD	41	<LOD	<LOQ	<LOD
P4	29217	<LOD	<LOQ	<LOD	69	<LOD	<LOQ	<LOD
P5	310	<LOD	<LOD	<LOD	25	<LOD	<LOD	<LOD
P6	1887	<LOD	70	<LOQ	142	<LOD	67	<LOD
P7	2235	<LOD	<LOQ	<LOD	34	<LOD	<LOD	<LOD
P8	154	<LOD	<LOD	<LOD	28	<LOD	<LOD	<LOD
P9	675	<LOD	<LOD	<LOD	34	<LOD	<LOD	<LOD
P10	131	<LOD	<LOD	<LOD	34	<LOD	<LOD	<LOD
P11	254	<LOD	<LOD	<LOD	31	<LOD	<LOD	<LOD
P12	315	<LOD	<LOD	<LOD	71	<LOD	<LOQ	<LOD
P13	1999	334	1005	112	543	<LOD	105	<LOD

[a] Quantified by automated electrochemiluminescent immunoassay (Elecsys NT-proBNP II, Roche Diagnostic).

CRediT authorship contribution statement

Alessio Lenzi: Writing – review & editing, Writing – original draft, Methodology, Formal analysis, Data curation. **Mariano De Cristofaro:** Writing – original draft, Data curation. **Denise Biagini:** Writing – original draft, Methodology, Data curation. **Silvia Ghimenti:** Methodology, Formal analysis, Data curation. **Silvia Armenia:** Methodology, Data curation. **Nicola R. Pugliese:** Visualization, Methodology, Investigation. **Stefano Masi:** Writing – review & editing, Funding acquisition, Conceptualization. **Fabio Di Francesco:** Writing – review & editing, Supervision. **Tommaso Lomonaco:** Writing – review & editing, Validation, Supervision, Resources, Project administration, Methodology, Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

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