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Quantification of Neuropeptide Y and Four of Its Metabolites in Human Plasma by Micro-UHPLC-MS/MS

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Abstract

Neuropeptide Y (NPY) is a 36-amino acid peptide circulating at a subpicomolar concentration participating in multiple physiological and pathological processes. NPY is prone to peptidolysis, generating metabolites with modified affinity for the five known receptors of NPY that mediate distinct effects. It is, therefore, crucial to distinguish each metabolite to understand the multiple functions of NPY. Since immunoassays are not able to distinguish NPY from its metabolites, we have validated a microliquid chromatography tandem mass spectrometry (micro-LC-MS/MS) assay for the quantification of endogenous NPY, NPY2–36, NPY3–36, NPY1–35, and NPY3–35 in human plasma. Sample preparation relies on immunoextraction in 96-well plates, followed by solid-phase extraction prior to micro-LC-MS/MS. The LLOQ ranged from 0.03 to 0.16 pM, intra- and inter-assay precision were <27% and trueness <22%. We determined reference intervals in 155 healthy volunteers and 40 hypertensive patients. We found that NPY3–36 is the main circulating peptide in resting conditions and that NPY and catecholamines are simultaneously increased during orthostasis. We also showed that the concentrations of NPY and its metabolites are similar in healthy volunteers and hypertensive patients. NPY is the prototype peptide that circulates in concentrations expected to be beyond instrumental capacities. We have been successful in developing a high-throughput specific and sensitive assay by including a deep knowledge of the physicochemical properties of these peptides to an efficient multistep sample preparation, and a

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ASSOCIATED CONTENT

Supporting Information

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Chemicals and standards, antibody coupling to magnetic beads, stability study, determination of reference intervals in healthy volunteers, statistical analyses for determining reference intervals, NPY and NPY metabolite levels during an orthostasis test in healthy individuals, and determination of NPY, NPY metabolite, and catecholamine concentrations in hypertensive patients (PDF)

The authors declare no competing financial interest.

micro-LC chromatography. We believe that our methodological approach opens the possibility to selectively quantify other endogenous peptides cleaved by peptidases whose concentrations are below 1 pM.

Graphical Abstract

Neuropeptide Y (NPY) is the most abundant peptide known in the brain.¹ It is involved in cardiovascular diseases and regulation,² food intake, obesity and metabolic diseases,³ anxiety disorders,⁴ and post-traumatic stress disorders.⁵ NPY stimulates at least five G protein-coupled receptors, called Y1, Y2, Y4, Y5, and Y6.⁶ It is coreleased with norepinephrine (NE) from nerve endings upon activation of the sympathetic nervous system and potentiates the post-synaptic action of vasoactive substances such as NE through the Y1 receptor.⁷ In contrast, Y2 receptors are located presynaptically and inhibit NE and NPY release in healthy individuals and animals when stimulated.⁸ Y5 receptors are involved in food intake and cardiovascular diseases.² Y4 receptors are activated by pancreatic polypeptide, a member of the NPY family.⁹

NPY (YPSKPDNPGEDAPAEDMARYYSALRHYINLITR-QRY-NH₂) is a 36-amino acid peptide prone to peptidolysis by multiple enzymes that may affect its biological function. Among peptidases displaying a high affinity for NPY, dipeptidyl peptidase IV (DPPIV) plays the primary role.¹⁰ By cleaving the N-terminal Tyr-Pro dipeptide, DPPIV generates NPY3–36, a metabolite without affinity for the Y1 receptor that is an Y2/Y5 receptor agonist.^{11–17} Since Y1 is the major vasoconstrictive receptor, this cleavage largely inactivates its action. Frerker et al.¹⁸ reported by MALDI-TOF mass spectrometry that NPY is exclusively degraded by DPPIV into NPY3–36 in EDTA-plasma but they did not provide quantitative data to assess kinetics of NPY cleavage efficiency with DPPIV and did not report other cleavage products of NPY. Other C-terminally truncated NPY metabolites have been observed in human serum, including NPY1–35, NPY1–34 cleaved by angiotensin converting enzyme (ACE), and NPY1–32.^{19,20}

We have found that the ex vivo incubation of NPY with serum resulted in its time-dependent cleavage into three main metabolites with the following order of efficacy: NPY3–36 >> NPY3–35 > NPY2–36.²¹ Specific inhibitors of DPPIV, plasma kallikrein

and aminopeptidase P prevented the production of NPY3–36, NPY3–35, and NPY2–36, respectively. Though Y1/Y2/Y5 receptor functions have been explored with selective agonists and antagonists with promising therapeutic intervention, there is still a lack of accurate selective quantification of NPY and its metabolites in biological fluids. Human plasma concentrations of immunoreactive NPY have been reported to range from 0.25 to 129 pM^{22–26} reflecting cross-reactivities of antisera directed against NPY with related peptides of the NPY family, other immunoreactive metabolites or even precursors of NPY. Though immunoassays recognizing the amidated form of the peptide are the most reliable technique to quantify biologically active NPY, they fail to discriminate the intact NPY from NPY2–36 and NPY3–36, which prevented studies to assess the function of Y1 versus Y2/Y5 receptors.²⁷

Mass spectrometry (MS) is a powerful tool that can distinguish peptides differing from one single amino acid and can be used as an alternative to immunoassays.²⁸ During the past decade, only one group reported the development of an MS assay for NPY and other peptides of the NPY family, unfortunately with insufficient sensitivity to measure endogenous NPY.²⁹ In the present study, we present the development and validation of a highly sensitive microliquid chromatography tandem mass spectrometry (micro-LC-MS/MS) assay for the quantification of subpicomolar concentrations of endogenous NPY and its metabolites NPY2–36, NPY3–36, NPY1–35, and NPY3–35 (later collectively referred to as “NPY metabolites”). We have established reference intervals in a healthy population (aged 0.1–61 years), and investigated NPY peptides levels in hypertensive patients and during orthostasis.

MATERIALS AND METHODS

See also Supporting Information for details on chemicals and standards, antibody coupling to magnetic beads, stability study, determination of reference intervals in healthy volunteers, statistical analyses for determining reference intervals, NPY and NPY metabolite levels during an orthostasis test in healthy individuals, and determination of NPY, NPY metabolite, and catecholamine concentrations in hypertensive patients.

Calibrants and QC Samples.

Heparinized plasma used for the preparation of calibrant or QC samples was treated by activated charcoal to eliminate or significantly decrease the endogenous NPY and NPY metabolite concentrations below the quantification levels. The stock solutions used to prepare the calibrants and QC samples were obtained from lyophilizates (70 pmoles) prepared in our lab from the provider’s lyophilizate. Stock solutions were diluted taking into account the peptide purity and content to obtain net peptide concentration in the working solutions. NG 0.8% and FA 0.1% were added to the solutions, and all dilutions were carried out in 5 mL Minisorp tubes (Nunc-Immuno, Thermo Scientific, Waltham, MA, USA).

The calibration curve was prepared in the charcoal-stripped plasma, previously supplemented with a solution containing proteases inhibitors. Six calibrant samples were prepared by spiking 10 to 0.05 pM NPY and NPY metabolites in the plasma. The actual

concentration of the calibrants is the sum of the spiked concentration and the residual concentration in the charcoal-stripped plasma (see Supporting Information Table 1).

The QC samples were prepared independently from calibrants, in the same charcoal-stripped plasma containing proteases inhibitors. Three QC levels were prepared: high (QCH) at 8 pM, medium (QCM) at 2 pM and low (QCL), at 0.25, 0.12, 0.20, 0.10, or 0.13 pM for NPY, NPY2–36, NPY3–36, NPY1–35, and NPY3–35, depending on the lower limit of quantification (LLOQ) of the corresponding peptide (see Supporting Information Table 2).

Sample Preparation.

The beads coated with NPY02 antibodies³⁰ were washed with NaCl using a magnet. An IS solution containing 250 pM of ¹³C, ¹⁵N-labeled NPY and 500 pM of ¹³C, ¹⁵N-labeled NPY3–36, 1–35, and 3–35 in a solution containing FA 0.1% and NG 0.8% was prepared. The sample (500 μ L of calibrant, QC, or unknown sample), 5 μ L of NG 10%, 10 μ L of the IS solution, and 10 μ L of washed beads were transferred to a 2 mL collection plate (Waters). The plate was sealed and placed on a thermomixer (MKR23, Hettich Benelux, Geldermalsen, the Netherlands) 1 h at 20 °C under agitation (850 rpm). After incubation, the plate was centrifuged 3 min at 822 RCF to focus the beads on the bottom of the wells, and 400 μ L of supernatant was discarded. The remaining plasma containing the beads was transferred in a protein crash plate (Interchim, Montluçon, France) previously rinsed with a NG 0.1% solution. The plasma was removed by filtration using a positive pressure device (Waters), and 2 $\times$ 300 μ L of a NG 0.1% solution was added to wash the beads. Finally, the beads were suspended in 150 μ L of an acetic acid 2 M solution and incubated 5 min on the thermomixer, at 20 °C under agitation (950 rpm), to eluate the peptides from the beads. The solution was filtered through the protein crash plate by positive pressure and collected in a new 2 mL plate, as well as additional 100 μ L of acetic acid 2 M solution to wash the beads. The eluate was incubated with 125 μ L of H₂O₂ at room temperature for 10 min.

The SPE extraction was performed on Waters Oasis HLB μ Elution 2 mg plates using positive pressure. The phase was first conditioned with 200 μ L of ACN and equilibrated with 200 μ L of acetic acid 2M. The sample was loaded onto the plate and washed with 200 μ L of acetic acid 2M. The analytes were then eluted in conical 700 μ L 96 wells plates (Waters) using 50 μ L of ACN 50%–FA 0.1%–NG 0.1%. The solvent was evaporated to dryness using a TurboVap 96 Concentration Evaporator (Biotage, Uppsala, Sweden) at 45 °C with a nitrogen flow rate set at 50. The dry residues were reconstituted with 20 μ L of NG 0.1%–FA 0.1%. The plates were sealed, shaken 5 min, and centrifuged 10 min at 2500 RCF.

Micro-UHPLC-MS/MS Analyses.

Separations were performed on a Waters Acquity UPLC M-class system (Waters, Milford, MA, USA) configured for trap and back-flush elution with an auxiliary pump (ASM), a trap unit (TVM) and an IonKey device. A 10- μ L volume of sample was injected in partial loop mode using a 10- μ L PEEKsil needle and a 20- μ L loop and trapped on a C18 column (Waters Acquity UPLC M-Class Symmetry C18 Trap, 100 Å, 50 mm $\times$ 300 μ m, 5 μ m) using MeOH 2%–FA 0.1% in water (v/v), delivered at 15 μ L/min by the ASM for 2 min. The separation was performed on a CSH iKey device (Waters iKey Peptide CSH C18 50 mm $\times$

150 μm , 1.7 μm) with a mobile phase composed of (A) FA 0.05% (v/v) in water and (B) FA 0.05%–TFE 4% (v/v) in ACN, delivered at 3 $\mu\text{L}/\text{min}$ with a linear gradient from 2% to 15% B over 1 min, then 22.5% B over 3 min, and finally 95% B over 0.5 min. The gradient was held at 95% B for 3.5 min followed by a column equilibration step at 2% B for 4 min. A solution containing water, MeOH, ACN, and IPA (1:1:1:1) was used as strong wash, whereas a solution of ACN 5% with 0.1% FA was used as weak wash and solution of ACN 5% was used as seal wash. The temperatures of the autosampler, the trap oven and the iKey oven were fixed at 10, 25, and 60 $^{\circ}\text{C}$, respectively. A Xevo TQ-S triple quadrupole mass spectrometer (Waters) equipped with an IonKey interface was coupled to the LC system. Analyses were performed in MRM mode using positive ionization (PI). The ESI conditions were set as follows: capillary voltage 4.0 kV, source temperature 150 $^{\circ}\text{C}$, cone gas flow 300 L/h, nebulizer gas 7.0 bar, nanoflow gas 0.2 bar, and collision gas flow 0.17 mL/min. The MRM transitions used for quantification are detailed in Table 1. The micro-UHPLC-MS/MS system was controlled by MassLynx Software version 4.1 SCN#901 (Waters), and data were processed using the TargetLynx module. A blank sample (ACN 20%–FA 0.1%–NG 0.1% in water) was injected after every sample. Quantification was carried out against the calibration curve (area/IS area) using linear regression with $1/x$ -weighing.

Analytical Validation.

The method was validated following the latest guidelines for bioanalytical validation from the European Medicines Agency (EMA)³¹ and the US Food and Drug Administration (FDA).³² Linearity was assessed via the r^2 , slope, and y -intercept measured on the back-calculated values of calibrants from five independent runs. Intra-assay precision was determined by immunoextraction and analysis of the QC samples in quintuplicate. Inter-assay precision and bias were evaluated on QC samples analyzed in triplicate on five separate runs. Recovery and matrix effects were studied as detailed by Matuszewski et al.³³ on seven plasmas from volunteers spiked with 0.4 and 8.0 pM NPY and NPY metabolites. LLOQ was determined by spiking plasma with known concentrations of peptides and analyzing quintuplicate samples. LLOQ was determined as the lowest concentration providing a CV < 30% and a bias < 20%. Carryover was estimated by injecting the lowest calibrant (0.05 pM spiked) and then the highest calibrant (10 pM spiked), followed by two blanks containing ACN 20%. This was repeated three times. Carryover was expressed as the ratio of the area measured for the blank on the area measured for the lowest calibrant.

Catecholamines Measurements.

Catecholamines (epinephrine (E) and NE) were analyzed by UHPLC-MS/MS as described previously.³⁴

Sample Collection.

Volunteers and patients were fasting and had abstained from coffee, tea, cigarettes, or alcohol during the 12 h preceding the sample collection. Blood samples were collected in ice-cooled 2.6 to 4.5 mL Li-heparin tubes containing the inhibitors. The samples were centrifuged within 30 min at 4 $^{\circ}\text{C}$, 2500 RCF for 10 min, and plasma was aliquoted and stored at -80°C until the assay.

RESULTS AND DISCUSSION

Assay Development.

The assay was developed by adapting a strategy used in our laboratory for the determination of peptides present at very low concentrations in plasma.³⁵ This strategy merges together three different approaches: (1) extraction of the peptides and their ¹³C,¹⁵N-labeled isotope IS from plasma (500 μ L) by monoclonal antibodies immobilized on magnetic beads in 96-well plates, (2) cleaning of the extract on reversed phase micro-SPE plates, and (3) drying and reconstitution of the sample in a minimum volume for MS analysis (20 μ L). The resulting extract was highly concentrated with targeted peptides whereas the maximum of interfering compounds was removed, thus enabling a significant increase in sensitivity and specificity. The throughput was significantly improved by filtering the plasma on 96-wells filter plates that retained the beads, instead of removing the beads using a classical magnetic tube holder with 16 positions only. The targeted peptides were quantified by UHPLC-MS/MS in MRM mode, using the transitions with the highest m/z values for precursor and product ions to reduce the background noise deriving from unspecific residual molecules of small molecular weight. As the complex sample preparation provided clean chromatograms without interferences, and as the correlation between both quantifier and qualifier transitions was excellent for thousands of analyses carried out for the development of the assay, only a quantifier transition was used. All peptides were systematically fragmented before the Proline 13 providing the y₂₄ fragment ion for NPY, NPY_{2–36}, and NPY_{3–36} or y₂₃ for NPY_{1–35} and NPY_{3–35}. This strategy yielded very good sensitivity (1–2 pM LOD, data not shown), although insufficient to detect endogenous NPY or NPY metabolites in plasma from healthy volunteers. The replacement of the UHPLC system by a micro-UHPLC instrument with a trap module and the IonKey interface enabled a 10- to 20-fold increase of the signal (Supporting Information Figure 1) and, therefore, the detection and quantification of endogenous NPY and NPY metabolites in the plasma sample from healthy volunteers (Figure 1). Micro-LC was preferred to nano-LC (flow rates 1–5 μ L/min versus 100–500 nL/min) because of its lower occurrence of the problems often encountered with nano-LC (high analysis times, clogging, spray instability), allowing to run series of >200 injections. Moreover, the integrated IonKey module eliminates the risk of having dead volumes and postcolumn leaks.

Particular attention was paid to eliminate nonspecific binding of the peptide to surfaces. Adsorption tests carried out on stock solutions as well as at each extraction stage showed a peptide loss up to 100% at picomolar concentrations on both polypropylene and glass surfaces. Nonetheless, no adsorption on the surfaces occurred in plasma, possibly because the numerous components of the plasma are competing with NPY to adsorb on the surfaces, as we observed for teriparatide.³⁵

All blood samples were collected in heparin tubes containing peptidase inhibitors to prevent ex vivo degradation of NPY. When inhibitors were added, the recovery of the five peptides after one night at 4 °C in plasma was within 85–115%. Conversely, recoveries of NPY, NPY_{1–35}, and NPY_{3–35} were 20–60% without inhibitors. However, plasma incubated at

37 °C for 2 h showed a decrease in NPY and NPY metabolite concentrations, whether the inhibitors were added or not. See Supporting Information Figure 2 for detailed results.

Analysis of stock solutions and plasma samples by MS in scan mode showed the presence of the oxidized form of the five peptides (mass +16), typical for peptides containing methionine (Met¹⁷). This oxidation may occur in vivo or during the sample preparation, but not in the source, as the retention time differs for the two forms. Moreover, the ratio of methionine vs methionine sulfoxide NPY forms measured in plasma was not constant for replicate samples but rather fluctuated with time, resulting in a lower precision. In vitro oxidation by H₂O₂ 10% for 10 min after immunoextraction provided 100% of peptides with methionine sulfoxide and significantly improved the precision. The absence of methionine sulfone formation was verified. Supporting Information Figure 3 shows the areas of methionine, methionine sulfoxide, and methionine sulfone forms of NPY after oxidation by H₂O₂ 10%.

Since no blank plasma with no detectable amounts of NPY was available, calibration curves and QC samples were prepared in plasma treated by activated charcoal. This treatment removed 90–98% of the NPY and NPY metabolites to obtain a plasma with concentrations of each peptide below the LLOQ values. The residual concentrations were taken into account for the preparation of calibrants and QC samples (see Supporting Information Table 2 for detailed values) and have to be evaluated for each new charcoal-stripped plasma batch.

Analytical Validation.

The response for all five analytes remained linear with $r^2 > 0.99$ over the whole calibration range. The mean r^2 , slope, and y-intercept measured in five independent runs for all five peptides are displayed in Supporting Information Figure 4.

The intra- and inter-assay precision (CV%) ranged from 5% to 27%, for all three studied concentration levels (see Table 2). An aberrant value for NPY1–35 at 0.1 pM in the intra-assay study was removed according to the Tukey test. The bias ranged from –22% to 22% for the three levels (see Table 2). Since there are neither certified reference materials nor reference methods for the specific quantification of NPY, bias was estimated based on QC samples prepared from stock solutions different from the ones used to prepare the calibrant samples. These specifications for precision and bias were considered acceptable considering the reference intervals that were determined (see below), in the absence of data on biological variation of NPY.

Recovery evaluated on seven plasmas at two concentration levels ranged from 16 to 52%, and showed a high variation with CV values up to 56% (NPY3–35, 8 pM) (see Supporting Information Table 3). Indeed, the yield is highly dependent on the matrix, as shown by the 2-fold higher yields measured in two plasmas compared to the mean value measured in the seven plasmas (data not shown), but this variation did not affect the final results due to the efficient correction by the IS (CV = 20%). Matrix effects values were highly dependent on the NPY species, ranging from 64% for NPY2–36 to 121% for NPY3–35, as recovery compared to water, but were constant for a given NPY (the CV calculated on the values of matrix effect of each peptide were all <10%, see Supporting Information Table 3). This low CV indicates an excellent reproducibility across different matrices.

LLOQ was determined at 0.12, 0.03, 0.16, 0.05, and 0.07 pM for NPY, NPY2–36, NPY3–36, NPY1–35, and NPY3–35, respectively, on the basis of a CV < 30% and a bias < 20%. These criteria were selected based on the NPY and NPY metabolite concentrations measured in plasma from volunteers.

Carryover initially was measured up to 10%, because of high nonspecific binding. The carryover was reduced below detectable levels by using strong solvents such as injector wash solution (see Materials and Methods) and by injecting a blank sample after every sample (ACN 20%-FA 0.1%-NG 0.1%), as already discussed elsewhere.³⁵

Sample dilution with charcoal-stripped plasma enabled the quantification of NPY and NPY metabolites at concentrations higher than the calibrant A or from sample volumes lower than 500 μ L without decreasing precision and accuracy, provided that the final concentrations were within the calibration range.

Stability.

The stability study was designed to verify the stability of the peptides in each step of the assay. Freeze (–80 °C) and thaw (22 °C) stability in plasma after three cycles showed recoveries of 87 to 111% at 5 pM and 72 to 126% at 0.5 pM NPY and NPY metabolites. Short-term stability in plasma at 5 and 0.5 pM showed recoveries of 87 to 111% and 75 to 129% after 4 h at 4 °C, and 77 to 105% and 52 to 123% after 4 h at 22 °C, respectively. Therefore, we recommend working on ice. Stability in plasma at –80 °C for long-term storage was verified using QC samples. Concentrations of all five peptides in QC samples measured for more than six months ($n = 11$) were stable as demonstrated by the CV 20% observed for QCM and QCH. QCL were not included because of the lower precision at these concentrations. Recoveries for extracted samples left in the autosampler for 72 h (postpreparative stability) were above 78% for all studied concentrations, except NPY3–35 at 5 pM (65%). Stability of peptides during a cycle of lyophilization - reconstitution has been assessed at 8 nM with recoveries between 85 and 115%; however repeated lyophilization is not recommended since recovery decreased to 63% after three cycles. The stability of lyophilizates stored at –20 °C with desiccant was shown by the stable areas measured in samples prepared from lyophilizates over more than one year. In conclusion, NPY and NPY metabolites in lyophilizates, in plasma or after extraction are stable for a suitable time to perform analysis in usual conditions.

Reference Intervals in Healthy Volunteers.

Samples from 155 healthy volunteers were used to determine reference intervals. Their NPY and NPY metabolite concentrations are detailed in Supporting Information Table 4. Circulating levels were below the LLOQ in 11, 76, 0, 61, and 7 of the 155 samples for NPY, NPY2–36, NPY3–36, NPY1–35, and NPY3–35, respectively. Their concentrations were set at half the LLOQ for reference intervals calculation. The Harris and Boyd's test did not highlight any difference between the values measured in women and men (>15 years old) for NPY, NPY3–36, NPY3–35, and NE. Conversely, the groups differed for E values, because of a larger distribution for men than for women. The test was not performed for NPY2–36 and NPY1–35 because of the large number of values below LLOQ. Age-specific

reference intervals were built using a partitioning method based on CLSI document C28-A3 (Supporting Information Table 5) and the LMS method (see scatter plots in Figure 2). Partitioning is the most widely recognized method for determining reference intervals, used in many studies such as the CALIPER study whose algorithm was used to build our reference intervals,³⁶ with one modification: the Tukey test was applied on partitions and not on the entire data set since the reference intervals are significantly different between age categories. For example, the 50th centile of NPY is at 1.56 pM for boys aged 9 years, and 0.32 pM for boys aged 10 years. To cope with this wide range of concentrations, the model should ideally contain a large number of partitions, which was not possible because of the limited number of volunteers enrolled in the study. Therefore, a second method, the LMS method, was used to obtain reference intervals without stratification. The LMS method was initially developed to build normalized growth curves³⁷ and was successfully used to get reference intervals for age-dependent biomarkers. Both methods provided similar reference intervals except for the 2.5th and 97.5th centiles, since the LMS method takes into account the extreme values that were removed by the Tukey test used for partitioning. Reference intervals for NPY2–36 and 1–35 were not calculated with the LMS method because of the high number of samples with concentrations < LLOQ. Subjects presenting with NPY, NPY3–36 or 3–35 values above the 90th centile of the LMS reference intervals did not show any difference in HR, SBP, or DBP (p -values = 0.10, see Supporting Information Table 6). NPY concentration correlated with NPY2–36 and NPY3–36, as well as with NE and E (r_s = 0.66, 0.59, 0.58, and 0.69, respectively, p < 0.001). Interestingly, these correlations were stronger for men (r_s = 0.76, 0.60, 0.66, and 0.74, p < 0.001) than for women, whereas only NPY was significantly correlated with NPY3–36 (r_s = 0.62, p < 0.001). NPY1–35 was correlated with NPY2–36 and NPY3–35 (r_s = 0.62 and 0.54, p < 0.001). NE and E were correlated with NPY, confirming for the first time that NPY and catecholamine synthesis are dependent for a given sympathetic tone. NPY and NPY metabolite concentrations were not correlated with HR, SBP, or DBP or any other physiological or demographic parameter. Detailed r_s values are presented in Supporting Information Table 7.

NPY and NPY Metabolite Levels during Orthostasis Test in Healthy Individuals.

The NPY, NPY metabolite, and NE levels measured in plasma from six young healthy individuals in supine position were in the 25th centile (Figure 3, blue circles), suggesting that they have sympathetic resting level lower than the average healthy population. After standing upright (Figure 3, red squares), NPY concentration immediately rose (+ 82%, p < 0.03), while NPY3–36 and NPY3–35 did not increase (+ 9%, p = 0.36 and +7%, p = 0.70). Catecholamine levels also increased simultaneously with NPY (NE = +147%, p < 0.001, and E = +184%, p < 0.02), in agreement with tilt tests showing a similar increase in catecholamine levels within the 2–6 min period after standing³⁸ but in contradiction with other results showing no increase for E.³⁹ These data support the hypothesis of cosecretion of catecholamines and NPY, but not of catecholamines and NPY3–36 and NPY3–35, thus indicating that NPY is degraded later by DPP4 and kallikrein²¹ after secretion from sympathetic granules. These findings contrast with those from Vlcek et al. reporting that NPY levels of approximately 40 pM (determined by a radioimmunoassay) are unaffected 3 and 10 min after orthostatic test in 8 healthy 30-year-old women.⁴⁰ The concentrations found by Vlcek et al. likely represent the total immunoreactive NPY that

are approximately 100-fold higher than the reference intervals determined for NPY and hindered the selective increase of the endogenous bioactive Y1 agonist. Our data clearly illustrate the critical importance of using specific MS assays to quantify a targeted biomarker in pathophysiological conditions.

NPY and NPY Metabolite Levels in Hypertensive Patients.

NPY and NPY metabolite concentrations were also measured in 40 hypertensive patients (Supporting Information Figure 5 and Supporting Information Table 8). The reference intervals obtained from the partitioning method were used as controls, since they are more adapted for comparison of groups of values for different ages than the reference intervals obtained from the LMS method. NPY concentrations observed in hypertensive patients were within the range found for nonhypertensive population ($p = 0.49$). In contrast, concentrations of NPY3–36 were significantly higher in hypertensive patients compared with reference intervals ($p < 0.0001$). The sampling protocol has been established with the assumption that half-life for NPY is below 5 min to ensure that basal levels of the peptide reflect the resting condition. For these reasons, blood samples were performed 15 min after the patients were in supine position to minimize postural sympathetic stimulation. We recently found that NPY3–36 has a longer half-life than NPY (24.6 vs 4.6 min, respectively, unpublished data). We hypothesized that hypertensive patients secreted more NPY than nonhypertensive controls before the 15 min rest and that NPY3–36 is a late marker of the sympathetic stimulated state occurring in these patients, while NPY has returned to baseline. This assumption also applies to NPY2–36 ($t_{1/2} = 28.3$ min, unpublished data) and NPY1–35, the levels of which are significantly higher in hypertensive patients compared with reference intervals. Catecholamines, whose half-lives are even shorter than NPY, returned to baseline after 15 min rest in the supine position and was found to be similar to healthy subjects.

The NPY/NPY3–36 ratio was lower and the NPY1–35/NPY3–35 ratio higher for patients with hypertension compared with reference intervals, because of their higher NPY3–36 and NPY1–35 levels (see Supporting Information Figure 5). Conversely, these two ratios were found to be well above the 90th centile in three hypertensive patients that had to be excluded from the statistical analysis since they are treated with linagliptin, a DPPIV inhibitor (blue crosses in Supporting Information Figure 5). These data corroborate the observation from Hubers et al., who showed that sitagliptin, another DPPIV inhibitor, decreases the degradation of NPY *in vivo*,¹⁷ and demonstrate the same effect for NPY1–35 cleavage into NPY3–35. These three patients had no differences in resting catecholamine concentrations, HR, SBP, and DBP compared with other hypertensive patients.

The NPY3–35/NPY3–36 ratio was not significantly different between healthy volunteers and hypertensive patients, indicating that kallikrein activity is not affected in hypertensive-treated patients, thus in contradiction with studies claiming that hypertension may have a link with a defect in the kinin formation by kallikrein.^{41,42}

CONCLUSION

This is the first method reporting the specific quantification of endogenous NPY and NPY metabolites circulating at subpicomolar levels. The method is based on a last generation MS

instrumentation and on a micro-LC system that generated a 10-fold increase in sensitivity while maintaining similar ease of use and throughput to normal LC. All steps of the sample preparation based on immunoextraction and then SPE were also optimized for sensitivity; for example, a high affinity monoclonal antibody was essential to produce a clean extract. However, these instrumental improvements and analytical optimizations were not sufficient to reach the expected sensitivity. The novelty of our approach relies upon the knowledge of the physicochemical, biochemical, and biological properties of the native peptide and its metabolites including the prevention of ex vivo enzymatic degradation, the search for low adsorption materials, the freezing of chemical degradation since the distribution of uncontrolled NPY oxidation states results in reduced sensitivity and precision, the production of blank matrix, etc. Neglecting one of these parameters results in the failure to develop the assay. These steps are not frequently taken into account because they are very time-consuming: more than 66% of the time spent for the development of our assay was dedicated to this optimization. Finally, when combined altogether, these methodological steps resulted in a robust and highly sensitive method that may be applied to other endogenous peptides.

The throughput of our assay was also optimized to be compatible with the needs of a laboratory performing clinical studies or routine assays. For example, the entire workflow has been developed in 96-well format, including immunoextraction which is performed on a 96-well filter plate to avoid clogging of the micro-LC, whereas it is usually performed on a magnet and requires tube by tube handling.

The validation of this MS assay showed LLOQ ranging from 0.03 to 0.16 pM for the five targeted peptides, enough sensitive to determine for the first time reference intervals in 155 healthy volunteers. We found that NPY is increased during orthostasis along with NE and E, strengthening the hypothesis that NPY is cosecreted with catecholamines during a sympathetic stress. Conversely, the other NPY metabolites that are not produced within the secretory granules result from the action of peptidases in the circulation and may target receptors expressed throughout the body to mediate delayed effects associated to Y2/Y5 stimulation.

Many endogenous peptides circulate in the same concentration range as NPY and are measured by immunoassays, with potential specificity issues. The strategy presented here may pave the way for the quantification of other peptides resulting from enzyme metabolism, improving the understanding of their functions.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Representative micro-UHPLC-MS/MS chromatograms of endogenous NPY and NPY metabolites (black arrows) extracted from the plasma from a healthy volunteer with the conditions detailed in the Materials and Methods section.

Figure 2.

Scatter plots showing the concentrations of NPY, NPY3–36, and NPY3–35 against age for female (red circles) and male (blue crosses) volunteers. The black lines represent the median values, and the gray areas the 2.5th, 10th, 25th, 75th, 90th, and 97.5th centiles.

Figure 3.

NPY, NPY3-36, NPY3-35, NE, and E levels measured during the orthostasis study. Three samples were collected with 2 min intervals from six young healthy volunteers in the supine position 15 min after the insertion of a venous catheter (blue circles). Three other samples were collected with 2 min intervals (red squares) as the volunteers stood upright and remained in the standing position. Data are presented as mean of the three measures with SD. Dotted lines show the 25th, 50th, 75th, and 97.5th centiles.

Table 1.

MRM Transitions, Dwell Time, Cone and Collision Voltages Used for Quantification

	precursor ion (<i>m/z</i>)	product ion (<i>m/z</i>)	dwell time (s)	cone voltage (V)	collision energy (eV)
NPY	715.50	754.90	0.030	60	18
NPY-IS ^a	719.50	759.90	0.015	65	18
NPY2–36	688.30	754.90	0.030	25	18
NPY3–36	672.20	754.90	0.030	20	14
NPY3–36-IS	676.20	759.90	0.015	55	14
NPY1–35	688.60	714.30	0.030	55	19
NPY1–35-IS	692.60	719.00	0.015	55	19
NPY3–35	645.10	714.30	0.030	30	15
NPY3–35-IS	649.10	719.00	0.015	30	15

^aUsed as internal standard for NPY and NPY2–36.

Inter-assay Precision and Bias Determined Using Five Runs of Triplicate Per Concentration Level and Intra-assay Precision and Bias Determined on One Single Run of Quintuplicates

Table 2.

	QC	target concentration (pM)	intra-assay precision, $n = 5$ (CV%)	bias, $n = 5$ (%)	inter-assay precision, $n = 5 \times 3$ (CV%)	bias, $n = 5 \times 3$ (%)
NPY	QCL	0.25	5	22	11	8
	QCM	2	6	0	6	4
	QCH	8	9	-10	9	-5
NPY2-36	QCL	0.12	14	-9	21	-14
	QCM	2	8	-22	7	-13
	QCH	8	6	-19	9	-15
NPY3-36	QCL	0.20	9	6	13	4
	QCM	2	8	3	8	9
	QCH	8	6	-10	8	1
NPY1-35	QCL	0.10	27 ^a	-4	26	5
	QCM	2	10	-7	8	-2
	QCH	8	5	-6	7	-2
NPY3-35	QCL	0.13	26	0	17	1
	QCM	2	9	-9	7	5
	QCH	8	9	-11	6	6

^aOne aberrant value was removed after Tukey test; CV was 47% with this value.