Multiplexed Assay to Quantify the PP-Fold Family of Peptides in Human Plasma Using Microflow Liquid Chromatography–Tandem Mass Spectrometry

Gemma Reverter-Branchat [a], Philippe J. Eugster [b], Christina Kuenzli,[c] Barbara Rindlisbacher,[c] Thomas Stauffer,[c] Christos T. Nakas [d,e], David Herzig,[a] Eric Grouzmann,[b,†] and Lia Bally[a,*,†]

BACKGROUND: Peptide Tyr-Tyr (PYY1-36), pancreatic polypeptide (PP1-36) and neuropeptide Y (NPY1-36) constitute the PP-fold family of peptides that is involved in metabolic regulation. Very low plasma concentrations and cleavage into active 3–36 fragments challenge bioanalytical assays used for the quantification of these peptides.

METHODS: We developed a multiplexed isotopic dilution assay to quantify PYY1-36, PP1-36, and NPY1-36 and their dipeptidyl peptidase-4 (DPP4)-derived metabolites PYY3-36, PP3-36 and NPY3-36. All peptides were immunocaptured from plasma using a monoclonal antibody and quantified by micro-ultra-HPLC-MS/MS. Blood samples from healthy volunteers were collected fasting and 30 min after nutrient stimulation. Method comparison was performed with commercial immunoassays.

RESULTS: Linearity was shown in the measured intervals ($r^2 > 0.99$). The lower limit of quantification (LLOQ) with a CV at 20% was 1.5 pM for PYY1-36 and PYY3-36, 3.0 pM for PP1-36 and PP3-36, 0.8 pM for NPY1-36 and 0.5 pM for NPY3-36. In all cases, intra- and inter-assay bias and imprecision were <21%. Pre-analytical stability required addition of a protease inhibitor cocktail. Physiological concentrations of PYY3-36, NPY3-36, PP1-36 and PP3-36 were above the LLOQ in 43% to 100% of the samples. PYY1-36 and NPY1-36 were above the LLOQ in 9% and 0% of the samples, respectively. Immunoassays showed higher concentrations of measurands and poor agreement when compared with micro-UHPLC-MS/MS.

CONCLUSIONS: The assay allowed for specific multiplexed analysis of the PP-fold family of peptides and their DPP4-cleaved fragments in a single sample, thereby offering new perspectives to study the role and therapeutic potential of these essential peptide hormones in health and metabolic disease.

Introduction

Peptide tyrosine tyrosine (PYY), pancreatic polypeptide (PP) and neuropeptide Y (NPY) are structurally closely related endocrine polypeptides that constitute the PP-fold family. This name derives from the shared hairpin loop structure referred to as the PP-fold. Members of the PP-fold family contain 36 amino acids with a large number of tyrosine residues (1). The PP-fold family of peptides is involved in metabolic regulation and is therefore of increasing interest for the understanding and treatment of obesity and related metabolic disorders (2).

The members of the PP-fold family are expressed at different concentrations in the gut–brain axis: PYY is predominantly synthesized and released by intestinal endocrine cells, PP is mainly found in pancreatic cells, and NPY is abundantly distributed in the central and peripheral nervous system (3, 4).

PYY, PP, and NPY exert their effects via G-protein coupled receptors, of which 5 have been identified: Y1, Y2, Y4, Y5, and y6 (5). Native peptide forms, namely PYY1-36, PP1-36 and NPY1-36, are cleaved by the ubiquitous enzyme dipeptidyl peptidase-4 (DPP4), removing the N-terminal dipeptide Tyr-Pro and generating the truncated fragments, PYY3-36, NPY3-36 and PP3-36. These cleavage products show distinct receptor affinity influencing their biologic action. This is best exemplified for PYY: the native PYY1-36 exerts protective effects on pancreatic beta-cells through Y1 stimulation (3, 6), but its cleavage product PYY3-36 selectively binds to Y2 in the hypothalamus inducing loss of appetite and weight loss (7).

[a]Department of Diabetes, Endocrinology, Nutritional Medicine and Metabolism, Inselspital, Bern University Hospital, University of Bern, Bern, Switzerland; [b]Laboratoire des Catécholamines et Peptides, Service de Pharmacologie Clinique, University Hospital of Lausanne, Lausanne, Switzerland; [c]Medics Laboratory AG, Bern, Switzerland; [d]Laboratory of Biometry, School of Agriculture, University of Thessaly, Nea Ionia Magnesia, Greece; [e]University Institute of Clinical Chemistry, Inselspital, Bern University Hospital, University of Bern, Bern, Switzerland.

*Address correspondence to this author at: Department of Diabetes, Endocrinology, Nutritional Medicine and Metabolism, Inselspital, Bern University Hospital, and University of Bern, Freiburgstrasse, 3010 Bern, Switzerland. E-mail lia.bally@insel.ch.
†These authors contributed equally.

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Reliable quantification of each specific member of the PP-fold family of peptides and insights into their degradative pattern is important for a deeper understanding of their physiology and therapeutic potential. However, their plasma concentrations in the low picomolar range and cleavage into active 3–36 fragments challenge bioanalytical assays.

Consequently, concentrations of PYY, PP and NPY reported in the literature vary greatly across different studies and often do not allow distinction between native and DPP4-cleaved products (8–10). This limitation is mainly due to the widespread use of immunoassays with well-known shortcomings related to nonspecific antibody binding, matrix effects, and a lack of internal normalization of the measurement system (11). As a result, measured concentrations of these peptides cannot be directly related to biological effects, thereby limiting pathophysiological understanding.

The potential of LC-MS/MS to quantify peptides is growing. In contrast to immunoassays, LC-MS/MS provides the advantage of high specificity, large capacity for multiplexing, and higher quantitative agreement across laboratories. LC-MS/MS-based approaches have been explored for the separate analysis of each of the PP-fold family member with varying degrees of success (9, 12, 13). One LC-MS/MS method for PYY lacked the analytical sensitivity to measure physiologic concentrations (12). Another method for isolated measurement of PP combined immunoaffinity enrichment with LC-MS/MS with acceptable analytical sensitivity (9). We have recently developed an LC-MS/MS assay for NPY and 4 fragments with good performance, but this assay was unable to simultaneously quantify PYY, PP and their DPP4-cleaved products (13).

To better understand the complex role of the PP-fold family of peptides in metabolic regulation and move drug discovery pathways (2, 14) forward, a novel approach that allows for the simultaneous quantification of all relevant peptides is desired. Our goal here was to develop and validate a multiplexed micro-ultra high pressure liquid chromatography-tandem mass spectrometry (micro-UHPLC-MS/MS) assay to simultaneously quantify each member of the PP-fold family, including their DPP4-cleaved products.

Materials and Methods

REAGENTS AND PREPARATION OF STANDARD SOLUTIONS

Details of the reagents are provided in the online Supplemental Material and Supplemental Table 1.

PREPARATION OF CALIBRANTS AND QUALITY CONTROL SAMPLES

Calibrants and QC samples at low, medium, and high concentrations (QCL, QCM and QCH respectively) were prepared by spiking charcoal-stripped blank plasma at the targeted concentration (see online Supplemental Tables 2 and 3) with peptide standards diluted in 1 mg/mL nonyl-β-D-glucopyranoside (NG)/1 mL/L formic acid.

SAMPLE PROCESSING

Calibrants, QCs and human samples (500 µL) were loaded in a 2 mL 96-well plate (Waters) with 10 µL of magnetic beads suspension (20 µg of antibody), 20 µL of stable ^{13}C, ^{15}N isotope peptides (IS) mixture and 5 µL of 0.1 g/mL NG in water (details provided in the Supplemental Material for IS and antibody-beads preparation). The plate was sealed and agitated at 850 rpm for 1 h at 20°C. The beads were recovered by centrifuging the plate at 2000*g* for 3 min and removing 400 µL of supernatant from each well. The remaining plasma containing the beads (approximately120 µL) was transferred to a protein crash plate (0.2 µm filter; Interchim), previously washed with 1 mg/mL NG in water. After filtering by positive pressure (Waters Positive Pressure-96 Processor), the beads retained on the bottom of the wells of the protein crash filter plate were washed twice with 300 µL of 1 mg/mL NG. For peptide elution, the beads were incubated in 150 µL of a 2 M acetic acid in ethanol:water (1:4) solution for 5 min at 20°C at 950 rpm.

The eluate was filtered into a clean 2 mL 96-well collection plate (Waters). Elution was repeated with 100 µL of the same solution. The eluate was treated with 125 µL of H_2O_2 solution (final concentration 10%) for 20 min at 20°C and diluted with 125 µL of 2 M acetic acid. The sample was loaded into a Waters Oasis® HLB µElution solid phase extraction plate conditioned with 200 µL of acetonitrile and equilibrated with 200 µL of 2M acetic acid and sequentially washed with 200 µL of 2 M acetic acid, 200 µL of 10 mg/mL ammonium sulphate and 200 µL of water. The peptides were eluted in a 700 µL collection plate (Waters) by 50 µL of a 450 mL/L acetonitrile/1 mg/mL NG/1 mL/L formic acid solution. After evaporation to dryness with nitrogen, extracts were reconstituted in 20 µL of a 1 mg/mL NG/1 mL/L formic acid solution. For analysis, the plate was sealed, shaken for 5 min, and centrifuged at 2500*g*.

MICRO-UHPLC-MS/MS

Separations were performed on an Acquity UPLC M-Class system (Waters) configured for trap and backflush elution with an auxiliary pump, a trap unit, and an IonKey system. Samples (10 µL) were injected in partial loop mode and trapped on a C18 column (Waters M-Class Symmetry C18 Trap, 50 mm × 300 µm, 100 Å, 5 µm) using 20 mL/L methanol/1 mL/L formic acid (v/

v) in water at 15 µL/min for 2 min. Peptides separation was performed on a CSH iKey column (Waters iKey Peptide CSH C18, 50 mm × 150 µm, 130 Å, 1.7 µm) at 50°C. Mobile phase A was 5 mL/L formic acid in water, and B was 5 mL/L formic acid/40 mL/L trifluoroethanol in acetonitrile. The starting flow rate was set at 1 µL/min with a linear gradient as follows: 0 min, 5% B; 17 min, 39% B; 19.5 min, 95% B with a flow increase to 3 µL/min; 23.0 min, 95% B; 23.5 min, 2% B. The flow was reduced to 1 µL/min at the end of the trapping.

The LC system was coupled to a Xevo TQ-S triple quadrupole mass spectrometer (Waters) equipped with an IonKey source. The instrument was operated in positive ion mode with capillary voltage 4.0 kV, source temperature 120°C, cone gas flow 300 L/min, nebulizer gas 7.0 bar, nanoflow gas 0.2 bar, and collision gas flow 0.17 mL/min. The analyses were performed in multiple reaction monitoring mode (see online Supplemental Table 4). The system was controlled by MassLynx software v4.1 SCN905 (Waters) and data was processed with the TargetLynx module. A blank sample was injected after every sample. Quantification was performed with a calibration curve using linear regression with 1/*x*-weighting.

ANALYTICAL VALIDATION

We followed the Bioanalytical Method Validation guidelines from the Food and Drug Administration (FDA) and the Clinical and Laboratory Standards Institute (CLSI) C62-A guidelines (15, 16). Peptide concentrations are expressed in pmol/L (pM). Intra-assay recovery and imprecision were determined by measuring five QC samples at low (QCL), medium (QCM) and high (QCH) concentrations within the same day. Inter-assay imprecision and recovery were determined by analysis of QCL, QCM and QCH samples (n = 28) over a 9-month period over 9 different runs. The lower limit of quantification (LLOQ) was established as the lowest concentration with <20% deviation from target and CV < 20%. For studies using clinical samples, we applied a LLOQ developed by repeated measurements (n = 26) in different blank plasma samples (n = 3) over 12 different runs at the lowest concentration with <20% deviation from target and imprecision CV < 30%. This allowed us to report peptide concentrations just below the pre-set LLOQ calculated with an imprecision of CV < 20%. Linearity was calculated using r^2, slope and *y*-intercept values obtained after back-calculated values from calibrants from 5 independent runs. Carryover was tested by injecting sequentially an LLOQ concentration sample, a sample at the highest calibrant concentration, then the interspersed blank, and a second blank sample containing 200 mL/L acetonitrile/1 mg/mL NG/1 mL/L formic acid 4 times. Carryover was calculated as the ratio of the area measured for the second blank to the area measured for the lowest calibrant. Spike recovery was obtained by comparing areas measured before and after extraction at QCL, QCM and QCH concentrations in sextuplicate. The matrix effect was estimated on 5 different plasmas spiked with analyte post-extraction compared to analyte injected into neat solvent solution at all points of the calibration curve. Both spike recovery and matrix effect analyses were performed by using the IS as representative of the unlabelled analyte (17, 18). Method linearity was assessed by diluting one sample spiked above the upper limit of quantification for all peptides, diluted 1:40 and 1:200, and each dilution analysed in quintuplicate.

STABILITY STUDIES

Susceptibility of PP-fold peptides to peptidolysis required stability characterization at multiple concentrations. Pre-analytical stability was evaluated by measuring triplicates of plasma from a healthy donor (endogenous concentrations) and plasma spiked with 1 pM of NPY1-36/3-36 and 20 pM of PYY1-36/3-36 and PP1-36/3-36 peptides. Extractions were done at time 0 and after having stored the plasma for 2 h and 4 h at two temperatures, 4°C and 20°C, and in the presence and absence of the protease inhibitor cocktail. Plasma stability after 1 month storage at −80°C was evaluated by triplicate analysis of QC samples at two concentrations (QCL and QCH). The freeze-thaw cycle effect was studied by triplicate analysis of samples at two concentrations (QCL and QCH) after 3 freeze/thaw cycles. Autosampler post-extraction stability was assessed by injecting extracts kept at 10°C at 2 concentrations (QCL and QCH) every 12 h up to 60 h. Recovery was calculated by comparison against freshly injected extracts. The effect of lyophilization on reference material was estimated by measuring in triplicate standard peptide solutions (10 nM) after 3 lyophilization cycles.

PEPTIDE ANALYSIS BY IMMUNOASSAY

Total PYY (EZHPYYT66K), total PP (EZHPP40K) and total NPY (EZHNPY-25K) enzyme-linked immunosorbent assay and PYY3-36 (PYY67HK) radioimmunoassay kits were from Merck Millipore and used according to the manufacturer's instructions. The plasma volume needed for each assay (duplicate) was 40 µL, 100 µL, 100 µL, and 200 µL, respectively. The PYY3-36 radioimmunoassay is described in the online Supplemental Material and immunoassay characteristics are provided in Supplemental Table 5. Commercial kit selection was based on highest use frequency in the scientific community and assay performance metrics.

PYY3-36 radioimmunoassay was included for direct comparison with the LC-MS/MS assay.

COLLECTION OF FASTING AND POSTPRANDIAL HUMAN BLOOD SAMPLES

Blood samples were collected from 13 fasted healthy volunteers (7 males and 6 females, aged 30 (10) years, body mass index 24.6 (3.3) kg/m^2, before and 30 min after standardized nutrient ingestion). The nutrient stimulus consisted of a high-fat chocolate bar (18 g of fat, 3.5 g of protein, and 27 g of carbohydrates) or isocaloric pure glucose (72 g) diluted in water, both ingested within 1 to 3 min. Ten subjects underwent sampling with both nutrient stimulations on separate days, whereas 3 subjects provided samples before and after receiving pure glucose only.

Blood was collected in pre-chilled heparin tubes with an in-house optimized protease inhibitor cocktail containing vildagliptin, aprotinin, E-64, leupeptin, pepstatin, actinonin, EDTA, 4-(2-aminoethyl)-benzenesulfonyl fluoride hydrochloride (AEBSF), orthophenanthroline and fasidotrilate (13). EDTA-blood samples with inhibitor cocktail were collected for total PP analysis by enzyme-linked immunosorbent assay to avoid potential interference with heparin following the manufacturer's instructions. Samples were centrifuged within 30 min at 4°C, 2500*g* for 10 min, and plasma was removed and stored at −80°C until analysis.

Sample collection was performed in accordance with the Helsinki Declaration and was approved by the Ethics Committee Bern (2019_00383).

DATA ANALYSIS

Fold-change peptide concentration significance was assessed using the Wilcoxon rank sum test. For clinical samples, LLOQ was established with a CV at 30% to attribute a peptide concentration for the majority of the samples quantified by LC-MS/MS (Table 1). The concentrations of the few samples for which measured peptide concentrations were below the LLOQ were replaced by a value of LLOQ/2 for statistical purposes (19). Variance component analysis for combined imprecision was performed with the R package "VCA" (20). Method comparisons were performed by means of Deming regression and Bland–Altman analyses using the R package "mcr" (21) and discarding values below the LLOQ. The software R version 4.0.2 (The R Foundation for Statistical Computing) and GraphPad Prism version 8.3.0 (GraphPad Software) were used for analyses and graphics.

Results

ASSAY DEVELOPMENT

Adsorption of the peptides on polypropylene or polyethylene surfaces was checked and efficiently prevented by

Table 1. LLOQ determined either with a CV at 20% (left), or with a CV < 30% (right), and number of values above these LLOQ measured in the samples from nutritional stimulus studies using mass spectrometry and immunoassay.[a]

		LLOQ with CV at 20%			LLOQ with CV <30%		
	Method[b]	LLOQ[c] (pM)	n >LLOQ, T_{0min}[d]	n >LLOQ, T_{30min}[d]	LLOQ (pM)	n >LLOQ, T_{0min}	n >LLOQ, T_{30min}
PYY 1-36	MS	1.5	2/23	12/23	0.5	18/23	22/23
PYY 3-36	MS	1.5	20/23	22/23	0.5	23/23	23/23
PYY 3-36	RIA	4.9[1]	3/21	5/21	NA	NA	NA
Total PYY	IA	3.5[1]	17/21	20/23	NA	NA	NA
PP 1-36	MS	3.0	10/23	18/23	1.28	16/23	22/23
PP 3-36	MS	3.0	15/23	22/23	1.24	19/23	23/23
Total PP	IA	2.9[e]	9/9	9/10	NA	NA	NA
NPY 1-36	MS	0.8	0/23	0/23	0.27	16/23	15/23
NPY 3-36	MS	0.5	23/23	23/23	0.27	23/23	23/23
Total NPY	IA	1.2[e]	4/23	18/23	NA	NA	NA

[a]The denominator represents the number of measured samples.
[b]Methods: MS, mass spectrometry, IA, immunoassay, RIA, radioimmunoassay.
[c]LLOQ with CV <20% is based on low QC concentrations, providing CV in the 10.8% to 20.8% range.
[d] T_{0min}: Sample collection at time 0 (before nutrien intake); T_{30min}: Sample collection time (after nutrient intake).
[e]LLOQ specified by the kit manufacturer with unknown imprecision, postulated here to be at CV < 20% and accuracy within 20%.
NA: Not applicable.

NG addition (13). As for NPY (13), the two methionine amino acids present on the PP1-36 and the PP3-36 sequence were prone to oxidation. The introduction of a mild oxidation step after immunocapture resulted in stable and constant formation of sulfoxide methionine sulfoxide (≥86%). Hence, multiple reaction monitoring (MRM) transitions for PP1-36 and PP3-36 incorporated the addition of 2 oxygen molecules (+16Da for each methionine). No additional oxidation of sensitive amino acids, such as histidine and tyrosine, was detected in H_2O_2-treated NPY by high-resolution mass spectrometry analysis (data not shown). Accordingly, apart from tyrosinamide in the C-terminal position and methionine oxidation, no other post-translational modifications have been described for PP-fold peptides in plasma (9, 13, 22). The final

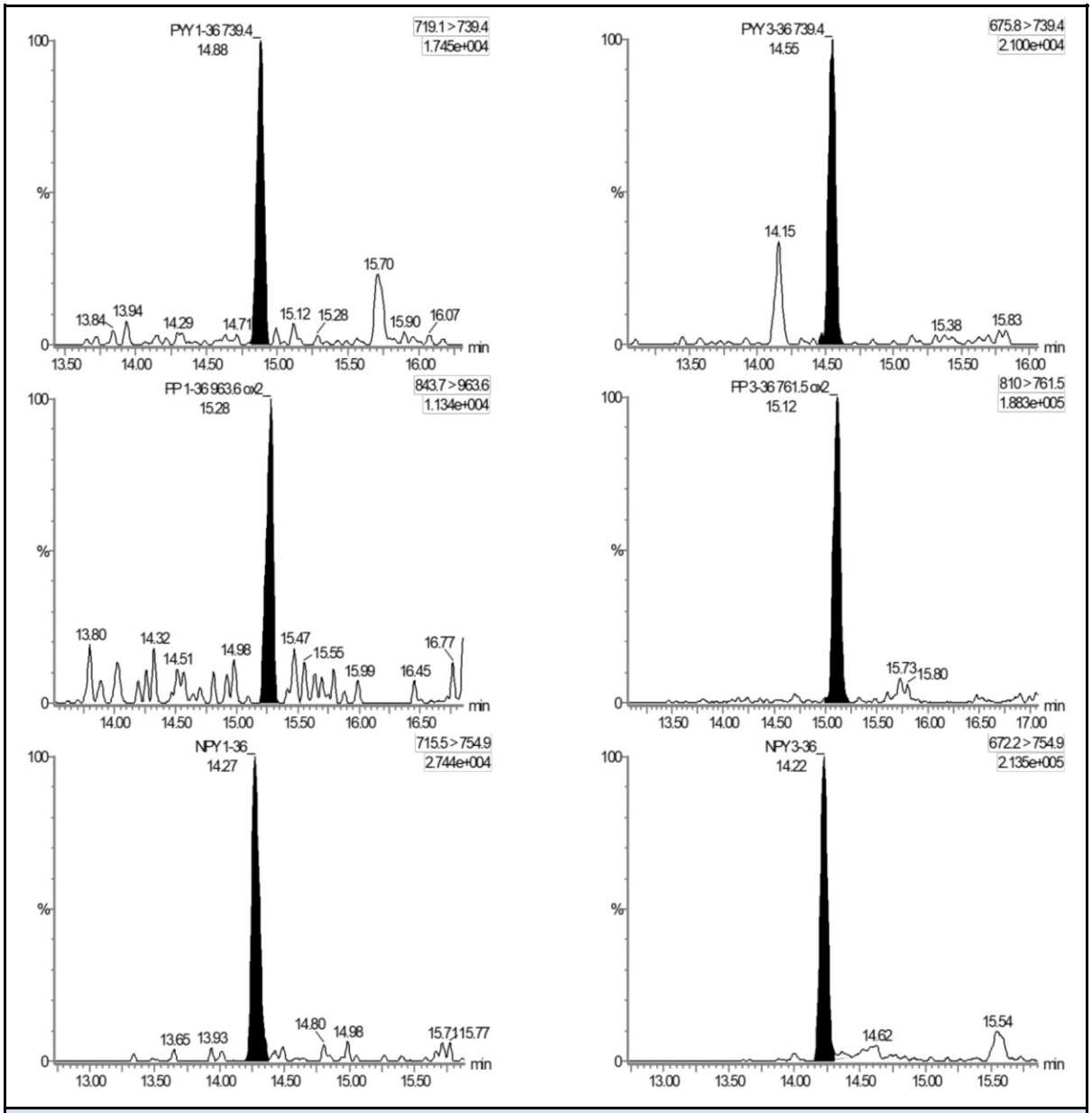

Fig. 1. Representative chromatograms of the quantifier transitions selected for each peptide measured in a plasma sample from a fasted healthy volunteer.

PYY (0.56 pM); PYY3-36 (0.99 pM); PP (1.71 pM); PP3-36 (1.57 pM); NPY (0.32 pM) and NPY3-36 (0.62 pM).

multiple reaction monitoring method included a quantifier and a qualifier transition for PYY1-36, PYY3-36, PP1-36, and PP3-36 peptides (see online Supplemental Table 4) whereas we used the quantifier transition previously validated for NPY1-36 and NPY3-36 (13).

Total analysis time per sample was 17.5 min. For each peptide, a representative chromatogram in a real sample at concentration near the LLOQ of the method is illustrated in Fig. 1. The microflow rate was reduced to 1 μL/min during peptide elution to obtain sufficient sensitivity for endogenous concentrations of the peptides (see online Supplemental Figs. 1 and 2).

ANALYTICAL VALIDATION

The LLOQ at CV < 20% was established at (pM) 1.5, 1.5, 3.0, 3.0, 0.8, and 0.5 for PYY1-36, PYY3-36, PP1-36, PP3-36, NPY1-36, and NPY3-36, respectively (Table 2). All peptides showed good linearity within the range of tested concentrations with $r^2 > 0.997$ (Table 2 and Supplemental Fig. 3). The intra- and inter-assay imprecisions showed a CV <21% for all peptides, with a combined imprecision <21% (Table 2 and Supplemental Table 6). Intra- and inter-assay recoveries of target concentrations exhibited an acceptable bias between -9% and 15% (Table 2), in accordance with method validation criteria. Linearity studied at 2 dilution factors (1:40 and 1:200) presented a recovery range of 75% to 95% for all peptides tested. Carryover for NPY (1%) was observed only with NPY in the blanks injected after samples with concentrations at the upper limit of quantification. To prevent this, a blank was interspersed between the samples that may have exceeded NPY concentrations of 10 pM. In contrast, no carryover was observed for PYY and PP.

Extraction recovery and matrix effects were measured using the stable isotopes of the peptides as previously described (18). Overall extraction recovery assessed on 7 different plasma samples ranged between 11% and 17% for NPY and PYY peptides (Table 2) but only reached 4% for PP1-36 and PP3-36. This was interpreted in the light of the lower affinity of the monoclonal antibody used for the PP peptides (23). The satisfactory recovery of the solid-phase extraction (SPE) step (e.g., 65% for NPY1-36) confirmed that the critical factor influencing analyte recovery was the immunoextraction. Matrix effect analyses revealed the presence of ion suppression effects ranging between 65% and 99%. Hemolysed samples resulted in high analyte loss (<10% recovery for NPY IS) and were therefore excluded from analysis. In contrast, the presence of lipids did not affect quantification.

STABILITY TESTING

Pre-analytical stability assessment showed that all peptides were stable for up to 4 h in the presence of the protease inhibitor cocktail at 20°C and 4°C, with a recovery between 77% and 117%. The absence of inhibitors, however, caused a fast reduction of NPY1-36, but not NPY3-36, concentration and a 30% decrease of PYY1-36 concentration within 2 h if

Table 2. Summary of the main validation parameters of the multiplexed micro-UHPLC-MS/MS method including all members of the PP-fold family of peptides and their corresponding DPP4-cleavage products.

Parameter		PYY1-36	PYY3-36	PP1-36	PP3-36	NPY1-36	NPY3-36
LLOQ (pM)		1.5	1.5	3	3	0.8	0.5
Calibration range (pM)[a]		0.5–64	0.5–64	1.28–128.28	1.24–64.24	0.27–16.02	0.27–19.13
Linearity	Intercept	−0.1194	0.2717	−0.1220	−0.2772	0.0367	0.0411
	Slope	1.0018	0.9972	0.9844	1.0196	1.0070	0.9902
	r^2	1.000	0.998	0.999	0.999	0.997	1.000
Intra-assay range	Imprecision (CV, %)	5–15	4–12	1–15[b]	4–17	2–11	3–4
	Bias (%)	10–14	−3 to 13	5–7	8–15	−1 to 7	−1 to 6
Inter-assay range	Imprecision (CV, %)	12–14	12–19	10–21	9–11	10–14	9–16
	Bias (%)	−3 to −8	−5 to −1	−5 to 1	−3 to −9	−5 to 1	−1 to −4
Matrix effect (mean ± SD, %)		74 ± 13	65 ± 18	68 ± 10	67 ± 10	93 ± 15	99 ± 16
Recovery range (%)		15–17	13–16	3–4	4	11–14	12–14

[a]A minimum of 6 calibrator concentrations were included for each peptide with a final concentration range established according to expected endogenous levels on actual blood samples (15).
[b]One QCL sample was measured using the second transition (843.7 > 723.0) due to suboptimal peptide recovery.

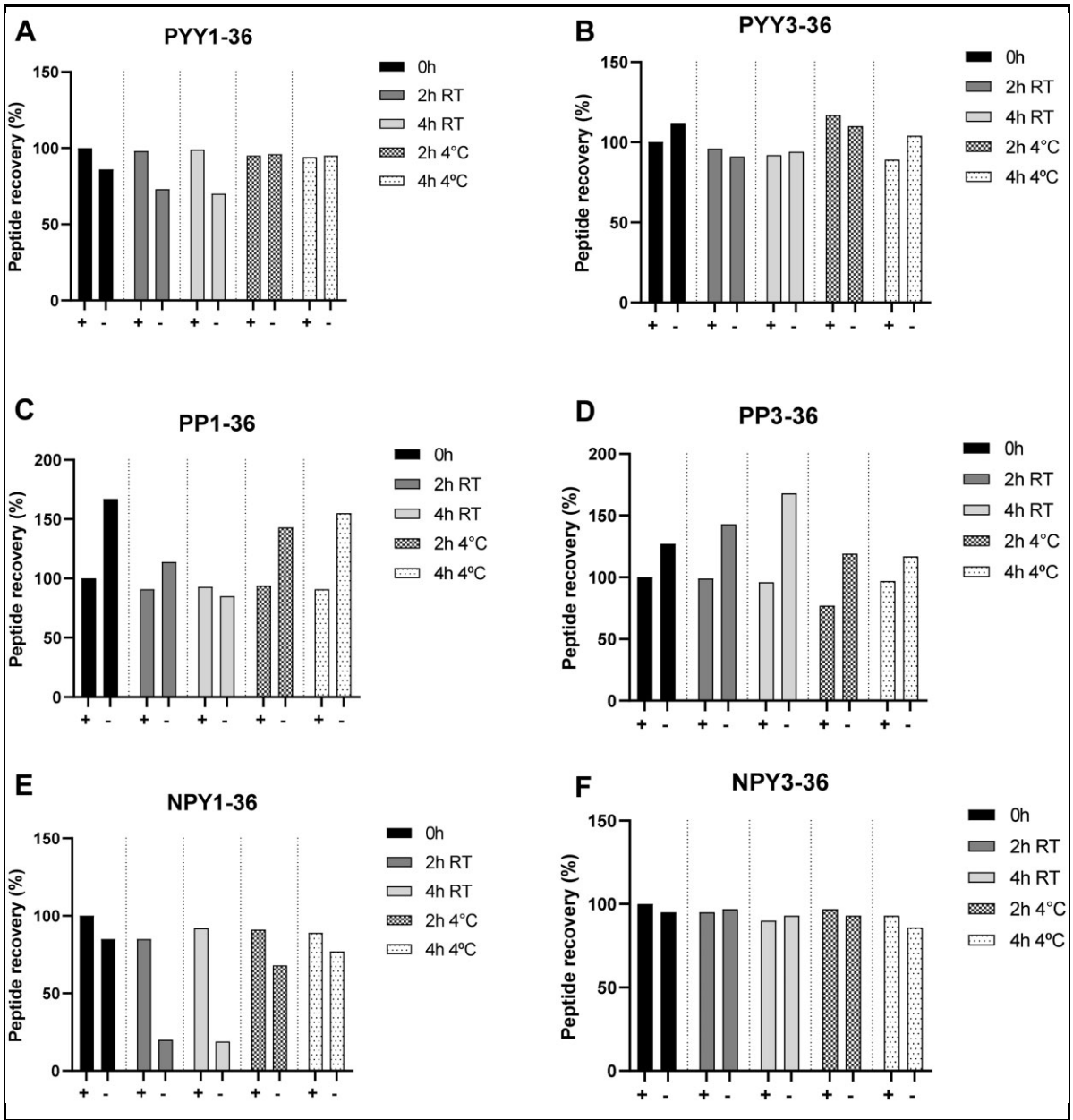

Fig. 2. **Pre-analytical stability of the peptides in plasma immediately measured after blood sampling and after being kept for 2 h and 4 h at room temperature and 4°C in the presence (+) or absence (−) of the in-house optimized peptide inhibitor cocktail.**

(A) PYY1-36; (B) PYY3-36; (C) PP1-36; (D) PP3-36; (E) NPY1-36; and (F) NPY3-36.

samples were not cooled at 4°C (Fig. 2). Conversely, PP1-36 and PP3-36 concentrations tended to increase in the absence of protease inhibitors suggesting a de novo production from PP prohormone caused by protease cleavage. Peptide recovery in plasma either after 1 month storage at −80°C or after 3 freeze/thaw cycles was >82% (see online Supplemental Table 7). Extract peptide recovery in the autosampler ranged between 73% and 125% after 60 h. Reference material was stable after 3 lyophilisation cycles of a 10 nM solution of standard peptides with a recovery range >94%.

Table 3. Fold-change increase in PP-fold peptides and their DPP4-metabolite concentrations measured by micro-UHPLC-MS/MS after mixed nutrient (n = 13) and isocaloric glucose (n = 10) stimulation.

Peptide	Relative change[a]	Mixed nutrient 95% CI	*P* value	Relative change[a]	Glucose 95% CI	*P* value
PYY1-36	1.8	[1.44–2.38]	<0.001	2.11	[1.26–6.52]	0.014
PYY3-36	1.34	[1.06–1.60]	0.032	1.48	[0.94–2.75]	0.106
PP1-36	8.2	[5.48–15.81]	<0.001	1.24	[0.47–3.49]	0.50
PP3-36	7.1	[4.81–10.00]	<0.001	2.04	[0.59–4.61]	0.49
NPY1-36	1.1	[0.74–1.69]	0.63	1.07	[0.45–1.71]	0.95
NPY3-36	1.02	[0.91–1.12]	0.84	0.94	[0.79–1.10]	0.49

[a]Data are median.

FASTING AND STIMULATED PEPTIDE CONCENTRATIONS

Measurements of samples from healthy volunteers showed between 1.8- and 8.1-fold nutrient-induced increase from baseline for PYY and PP peptides (Table 3). PYY1-36 and PYY3-36 increased following both mixed nutrient and glucose stimulation, whereas PP1-36 and PP3-36 concentrations increased only following mixed nutrient ingestion (Fig. 3). Mean fasting concentrations of native peptides and 3–36 fragments were 1.1 and 3.7 pM for PYY and 10.1 and 14.7 pM for PP. Postprandial concentrations were 1.7 and 5.1 pM for PYY and 17.3 and 24.6 pM for PP, respectively. Conversely, concentrations of NPY1-36 and its 3–36 fragment were not modulated by nutrient stimulation and showed mean concentrations of 0.4 and 1.3 pM, respectively. The percentage of values below LLOQ for PYY1-36, PP1-36, PP3-36, and NPY 1–36 were 13%, 17%, 9%, and 33%. Concentrations of PYY3-36 and NPY 3–36 were all above the LLOQ (Table 1).

METHOD COMPARISON

Fasting and postprandial plasma samples were also analyzed by immunoassays. For PYY3-36 and total NPY, the proportion of values below the respective LLOQ was >50% (Table 1). Only 2% of measured samples yielded PYY3-36 concentrations higher than the LLOQ.

Method comparison was performed by contrasting the sum of 1–36 and 3–36 peptides determined by LC-MS/MS with total concentrations assessed using immunoassay (see online Supplemental Fig. 4). Head-to-head comparison of PYY 3-36 was precluded due to lack of analytical sensitivity of the radioimmunoassay method. Resulting Deming regression equations were PYY MS = 2.43 + 0.2 * PYY immunoassay ($r = 0.60$); PP MS = −24.48 + 0.77 * PP immunoassay ($r = 0.37$); and NPY MS = 1.21 + 0.07 * NPY immunoassay ($r = 0.25$) demonstrating poor agreement between the 2 methods. Corresponding Bland-Altman plots are shown in online Supplemental Fig. 4.

Discussion

The results and implications of the present work are twofold. First, we developed a UHPLC-MS/MS isotopic dilution assay to quantify both intact and truncated members of the PP-fold family with a high degree of analytical specificity presently lacking with available immunoassays. Second, our approach allows for multiplexing of the target peptides, thereby offering advantages in convenience as well as versatility.

The presented assay measures PYY1-36, PP1-36, NPY1-36 and their DPP4-cleaved fragments in human plasma meeting the bioanalytical method validation (15). The LLOQ at 20% CV for all 6 peptides ranged between 0.5 and 3.0 pM, resulting in an assay at least as analytically sensitive as most available immunoassays. Using this LLOQ, the 6 peptides were quantified in 47% (130/276) of the clinical samples only. Since a large proportion of subjects exhibited peptide concentrations just below the LLOQ, adjusting the CV of the LLOQ to 30% allowed for a 3-fold decrease of LLOQ and hence higher yield of quantifiable samples (87%) at the expense of a slightly higher imprecision at these critical concentrations (CV 30% instead of CV 20%). Of note, the higher imprecision did not preclude the proper interpretation of the results since the clinical intervention raised these concentrations by factors ranging from 1.8 to 8.1. In contrast, the detection of PYY3-36 and NPY3-36 quantified by immunoassay failed in >80% of cases.

Particularly novel is the assay's ability to quantify PYY1-36 and PYY3-36 at physiological concentrations and with full analytical specificity. Both peptides appear to be involved in the pathophysiology of highly prevalent diseases such as obesity and diabetes. Thus, the exploration of these candidates across the entire concentration range is relevant for an improved pathophysiological understanding as well as the development of targeted therapies (3, 24).

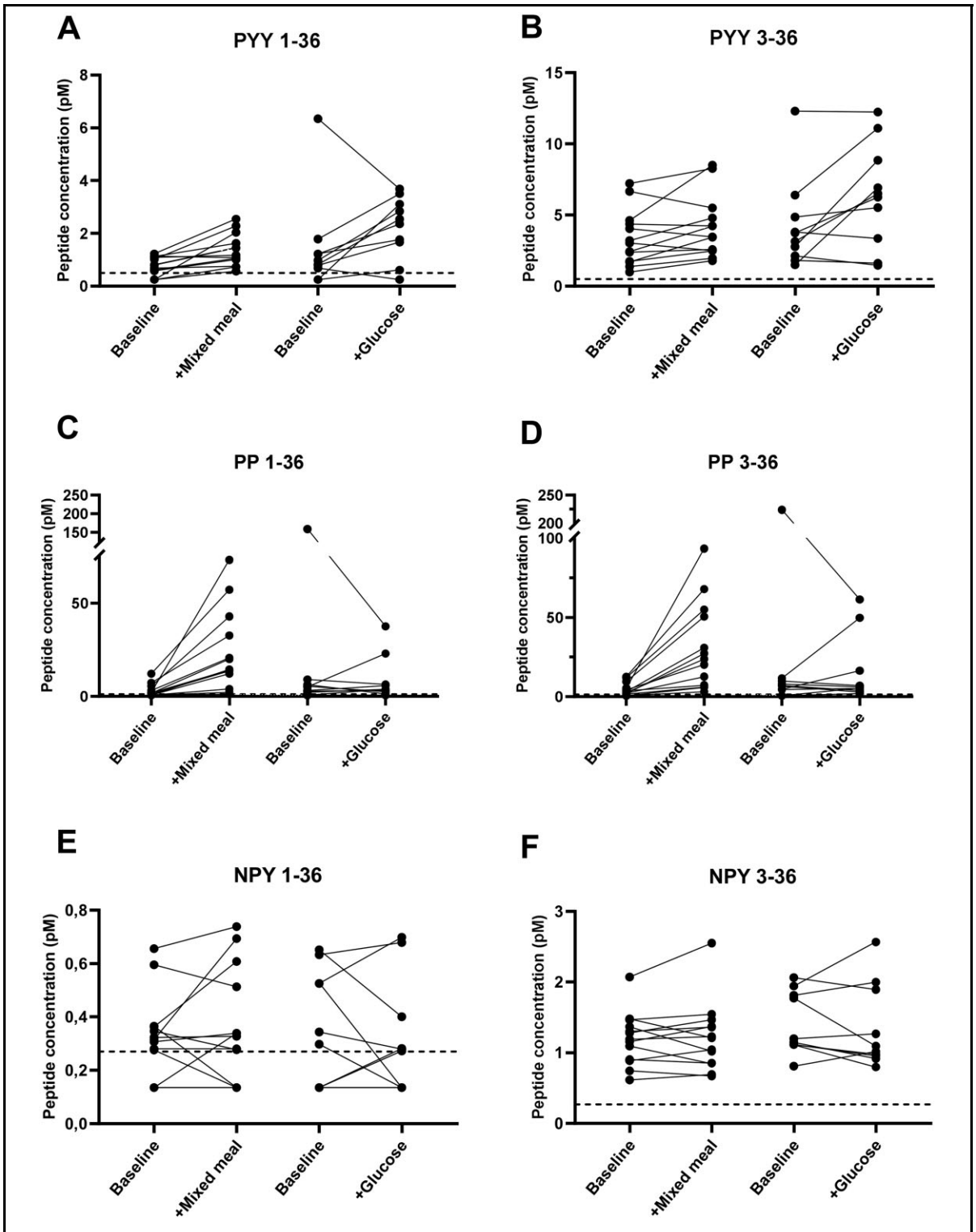

Fig. 3. Peptides concentrations before (t = 0 min) and after intake (t = 30 min) of a high-fat nutrient-mix or a pure glucose stimulus.

(A) PYY1-36; (B) PYY3-36; (C) PP1-36; (D) PP3-36; (E) NPY1-36; and (F) NPY3-36. The dotted line represents the corresponding LLOQ.

The greater capacity for multiplexing provides a further advantage of LC-MS/MS over immunoassay and permits high versatility on a per sample basis (25). Besides the assay's ability to differentiate all members of the PP-fold family, the proposed assay can be expanded by addition of further capture antibodies (e.g., against proglucagon-derived peptides) (18). This will allow for the simultaneous profiling of additional peptide hormones relevant for the study and treatment of metabolic diseases (26).

The strength of this work consists of the development of a rigorous analytical workflow mastering various challenges such as the low picomolar endogenous concentrations of the target peptides, their proneness to adsorption as well as their degradation by peptidases and oxidation (9, 27–29). By combining targeted immunoaffinity enrichment of the sample, SPE and microflow-liquid chromatography (LC) with the use of stable isotope-labelled IS and MS detection, our assay achieved high sensitivity while providing unparalleled specificity. Such hybrid methods are increasingly being used, particularly in drug discovery and early stages of clinical development, enabling biomarker measurement previously considered unattainable (27). A further strength is the assay's ability to multiplex, which, apart from the higher versatility, enables increased throughput, decreased costs and volume needs on a per sample basis. Lastly, intact peptide quantification (top-down strategy) excludes issues associated with peptide/protein analysis after proteolytic digestion (25).

The assay was successfully validated according to current guidelines and showed superior performance compared to the few previously published quantitative LC-MS/MS methods for PYY and PP (9, 12) as well as commonly used immunoassays. Analysis of fasting and postprandial plasma samples from healthy volunteers demonstrated that the proposed assay is capable of quantifying physiologically relevant concentrations of the target peptides, including nutrient-specific effects on secretory profiles. Although we applied the assay to the lower range of expected peptide concentrations in humans to reflect physiological conditions, its good linearity also supports its usefulness at supraphysiological concentrations for various clinical application (e.g., biomarker of neuroendocrine tumours) (30). Respective confirmatory biomarker qualification studies have yet to be performed. Since future clinical decision points are expected to be in the high rather than low concentration ranges, the comparably higher CV of 30% at LLOQ was deemed acceptable.

We also acknowledge limitations. From an analytical perspective, transferability of the proposed method is limited to laboratories with similar sophisticated equipment. Such high-performance instrumentation was, for example, necessary to offset the comparably low recovery of PP. In addition, the use of an antibody introduces extra work steps and dependency on a nonspecific, high-affinity monoclonal antibody. In our experience, an antibody-based analyte enrichment is required when plasma peptide concentrations are below 2 to 3 pM (13, 27, 31). Indeed, endogenous PYY quantification failed when using the same instrumental setup without immunoaffinity enrichment (32). However, more sensitive MS systems (e.g., up to 10-fold higher) may circumvent the use of antibodies and microflow-LC in future and favour, at the same time, analytical procedures with lower volume plasma requirements. Additionally, advanced instrumentation, ideally in terms of both performance and simplicity, may further shorten run times (e.g., to 6 to 10 min), which is important for high throughput.

Supplemental Material

Supplemental material is available at *Clinical Chemistry* online.

Nonstandard Abbreviations: PYY, peptide YY; PP, pancreatic polypeptide; NPY, neuropeptide Y; DPP4, dipeptidyl peptidase-4; LLOQ, lower limit of quantification; QCL, QC sample, low concentration; QCM, QC sample, medium concentration; QCH, QC sample, high concentration; NG, nonyl-β-D-glucopyranoside; IS, stable ^{13}C, ^{15}N isotope peptides.

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