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Validation of an LC-MS/MS Method Using Solid-Phase Extraction for the Quantification of 1-84 Parathyroid Hormone: Toward a Candidate Reference Measurement Procedure

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BACKGROUND: Parathyroid hormone (PTH) measurement is important for patients with disorders of calcium metabolism, including those needing bone-turnover monitoring due to chronic kidney disease–mineral bone disorder. There are currently 2 generations of PTH immunoassays on the market, both having cross-reactivity issues and lacking standardization. Therefore, we developed an LC-MS/MS higher-order method for PTH analysis.

METHODS: The method was calibrated against the international standard for 1-84 PTH (WHO 95/646). Antibody-free sample preparation with the addition of an isotope-labeled internal standard was performed by solid-phase extraction. Extracts were analyzed by LC-MS/MS. EDTA-K_2 plasma was used throughout the development and validation. Bias and uncertainty sources were tested according to ISO 15193. Clinical Laboratory Standards Institute guidelines and reference measurement procedures were consulted for the design of the validation. Patient samples and external quality controls were compared between LC-MS/MS and 2 third-generation immunoassays.

RESULTS: The method was validated for 1-84 PTH from 5.7 to 872.6 pg/mL. The interassay imprecision was between 1.2% and 3.9%, and the accuracy ranged from 96.2% to 103.2%. The measurement uncertainty was <5.6%. The comparison between LC-MS/MS and the immunoassays showed a proportional bias but moderate to substantial correlation between methods.

CONCLUSIONS: This LC-MS/MS method, which is independent of antibodies, is suitable for a wide range of PTH concentrations. The obtained analytical performance specifications demonstrate that development of a reference measurement procedure will be possible once a higher order reference standard is available.

Parathyroid hormone (PTH) is an 84 single-chain amino acid peptide produced by the parathyroid glands and released into the circulation when the plasma-ionized calcium concentration decreases. PTH binds to bone and kidney cells expressing the type 1 PTH/parathyroid hormone–related peptide receptor to exert its biological activity. In the kidney, PTH stimulates the reabsorption of calcium, stimulates the activity of 1-alpha hydroxylase to produce 1,25 dihydroxyvitamin D, and decreases phosphate reabsorption (1). PTH controls bone remodeling and calcium and phosphate homeostasis by acting on chondrocytes, osteoprogenitors, osteoblasts, and osteocytes (2).

Once in circulation, the half-life of 1-84 PTH is 2 to 4 min (3), and the peptide is metabolized in the liver. This pathway leads to the production of truncated forms such as large C-terminal or mid-truncated PTH fragments. Of note, such fragments are also secreted by the parathyroid glands (4–6). These fragments have a longer half-life than 1-84 PTH itself and represent approximately 15% to 30% of the total PTH in healthy individuals. Since these fragments are cleared by the kidneys, they accumulate in the blood of patients suffering from chronic kidney disease (CKD), and can represent up to 70% to 80% of the total PTH (7, 8).

In daily practice, PTH is measured for the diagnosis of primary and secondary hyperparathyroidism (9). It is also measured in patients with CKD and patients

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undergoing hemodialysis (DIA) for the management of CKD–mineral bone disorder, a frequent complication of advanced renal disease leading to high- or low-turnover bone diseases and/or vascular or soft-tissue calcification. This measurement is achieved through immunoassays using a pair of antibodies targeted against the C- and N-terminal parts of the peptide. Two generations of immunoassays are currently present on the market. Intact (or second-generation) PTH assays have been used since 1987 (10). In these assays, an N-terminal antibody is targeted against the 13–24 N-terminal part of the peptide (except the Roche intact PTH assay in which the N-terminal antibody recognizes an epitope located further from the N-terminal). In addition to 1-84 PTH itself, these assays have been shown to recognize a family of large C-terminal fragments referred to as non-(1-84) PTH (11), which explains their overestimation of PTH in the context of secondary hyperparathyroidism (12, 13). In 1999, third-generation immunoassays (also called whole or bioactive PTH assays) appeared on the market. Third-generation assays have the N-terminal antibody directed toward the first 4 amino acids of the peptide and are presented as highly specific for the 1-84 peptide due to their absence of cross-reactivity with the non-(1-84) PTH (14, 15). Both assay generations have also been reported to cross-react with oxidized PTH, a posttranslational modified variant supposedly produced in patients undergoing strong oxidative stress, such as DIA patients (16). Third-generation PTH assays also cross-react with another posttranslational modification variant called amino-PTH, a form of nontruncated PTH (17), which is overproduced in parathyroid carcinoma (18, 19) and severe types of parathyroid hyperplasia in patients undergoing DIA (20). Discrepancies between the different PTH assays can partly be explained by the aforementioned different cross-reactivities, especially in patients in which modified PTH forms tend to accumulate (8, 19).

In addition to immunoassays, higher-order techniques, such as LC-MS/MS, have been described for PTH measurement. Such methods have the potential to become reference measurement procedures (RMPs), as long as they satisfy the required analytical performance specifications (APS) and are able to overcome cross-reactivity issues with fragments or posttranslational modification forms. To date, 2 mass spectrometry-based methods have been described using the proteolytic digestion of the peptide (21, 22). Protein digestion generates many fragments that can lead to a loss of information in the case of modifications on the protein sequence. One other published method measures 1-84 PTH without digestion by high-resolution mass spectrometry (23). Although these 3 methods may provide clinical insight into patients with hyperparathyroidism, they present rather high limits of quantification of >30 pg/mL, which approximately corresponds to higher limit of 1-84 PTH normal range, according to some immunoassays (24, 25). Additionally, these assays rely on an antibody-dependent sample preparation, which increases the assay complexity and potential sources of variability.

We describe here the validation of an LC-MS/MS method with an antibody-free sample preparation, calibrated against an internationally recognized standard (WHO 95/646), for the precise quantification of 1-84 PTH, suitable for a wide range of PTH concentrations.

Materials and Methods

MATERIALS AND REAGENTS

LC-MS grade acetonitrile (ACN), methanol, and water were purchased from Biosolve B.V. DMSO >99.9% purity was purchased from Sigma–Aldrich (MilliporeSigma). LC-MS grade formic acid was purchased from Fisher Chemicals. Bovine serum albumin (Sigma–Aldrich) was used when diluting PTH standard solutions to help reduce nonspecific binding to laboratory consumables. The adsorptive loss assessment is presented in the online Supplemental Material file. Protein LoBind Eppendorf (Eppendorf SE) vials were used throughout the sample preparation.

Fresh horse plasma, used as a surrogate matrix, was donated by the Department of Endocrine Reproduction of the Veterinary Faculty of the University of Liège. For validation, PTH-blank human EDTA plasma (as determined by our method and immunoassays) was pooled from leftover samples from patients presenting with idiopathic hypoparathyroidism. Evolute Express weak cation exchange (10 mg) solid-phase extraction (SPE) 96-well plates were purchased from Biotage. AcroprepAdv 0.2-µm wwPTFE filters from Pall Corporation were used for filtering. WHO PTH 1-84, human, recombinant (First International Standard 95/646), containing 98.52 µg of PTH 1-84, was purchased through the National Institute for Biological Standards and Control. A 1-84 PTH peptide 15N stable isotope label was used as an internal standard (SIL-IS), acquired from Novus Biologicals, LLC. 7-84 PTH was purchased at Bachem AG.

STANDARD, CALIBRATION, AND QUALITY CONTROL SAMPLES

The PTH 1-84 standard and SIL-IS were reconstituted with a 10% ACN, 0.1% formic acid, and 1 mg/mL bovine serum albumin solution at 99.81 µg/g and 10 µg/mL, respectively. Aliquots of 40 µL were transferred to 1.5-mL Eppendorf vials, centrifuged for 30 s at 5000*g*, and frozen at −80°C. Preparation of 9-point calibration curve and quality control (QC) samples is described in Supplemental Material file. The calibration curve was

built from the peak area ratio of the native standard to the IS using weighted (1/x) linear least squares regression.

SAMPLE PREPARATION

One thousand microliters of leftover EDTA-K_2 plasma samples (Becton, Dickinson and Company) were aliquoted into 2.0-mL vials, centrifuged for 30 s at 5000*g*, and frozen at –80°C for less than a week until analysis. For each batch, calibrators, QCs, and samples (thawed at 5°C) were spiked with 20 μL of SIL-IS at 5.0 ng/mL and vortexed for 10 s. After 10 min of benchtop equilibration, they were diluted with 1000 μL of a 0.1 M NaH_2PO4 and 10% ACN solution adjusted to pH 7.0 (buffer), vortexed, and left 5 min for equilibration on the benchtop. Water in all aqueous solutions was LC-MS grade. The SPE procedure is detailed in Fig. 1. A vacuum not exceeding –5 kPa was applied in every step of the SPE until a flow of approximately 1 drop/s was obtained.

ETHICS

Remnant samples only were used in this study. No specific approval was requested to the CHU de Liège Institutional Review Board since a leaflet including the following statement is given to all admitted patients: "According to the law of the December 19, 2008, any left-over of biological material collected from patients for their standard medical management and normally destroyed when all diagnostic analysis have been performed, can be used for validation of methods." The law authorizes such use except if the patient expressed an opposition when still alive (presumed consent). Written informed consent for participation was not required for this study in accordance with the Belgian national legislation and the institutional requirements.

LC-MS/MS ANALYSIS

Separation and quantification were achieved by using a Nexera X2 UPLC from Shimadzu Corporation coupled to a QT6500 mass spectrometer from ABSciex operated at unit resolution for both quadrupoles. The column used was an Acquity® UPLC Protein BEH C4 Column 300 Å, 1.7 μm, 2.1 mm×100 mm from Waters Corporation maintained at 70°C. The mobile phase composition was A (water) and B (ACN) both containing 0.4% formic acid and 5% DMSO. The injection volume was 40 μL. The binary gradient profile is shown in online Supplemental Table 1 and Supplemental Fig. 1. Samples were kept in an autosampler at 5°C. Optimized multiple reaction monitoring and mass spectometry parameters are displayed in Supplemental Tables 2 and 3.

METHOD VALIDATION

The method was thoroughly tested for sources of bias and uncertainty to satisfy the requirements of ISO

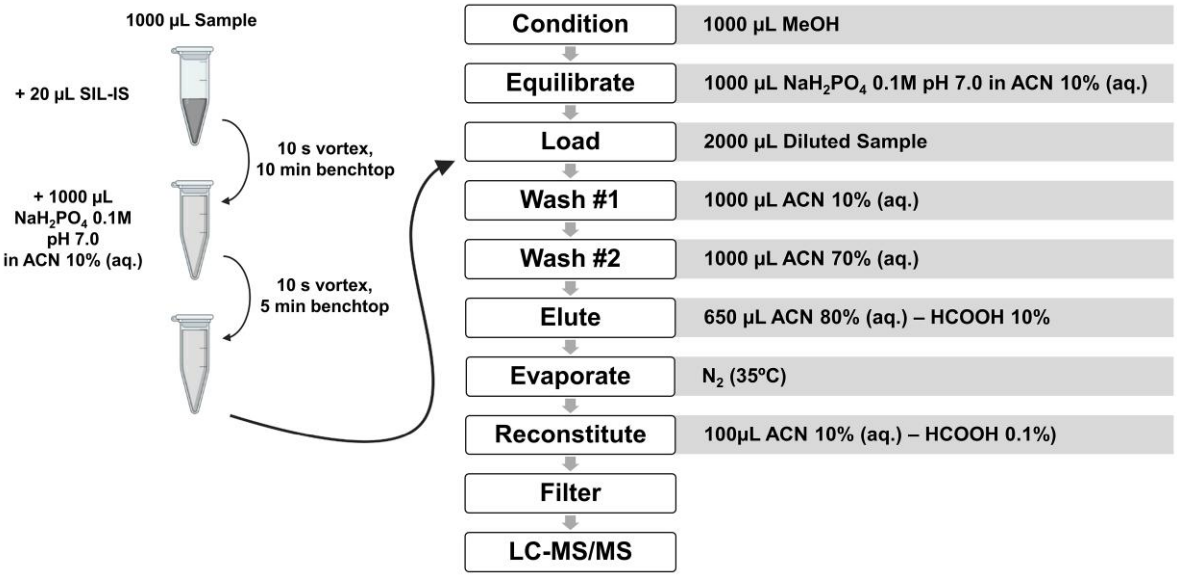


Fig. 1. Sample preparation scheme. SPE was conditioned with 1000 μL of methanol followed by 0.1 M NaH_2PO4 solution. 2000 μL of diluted samples were loaded and washed by 1000 μL of 10 and 70% ACN(aq). Elution required 650 μL of 80% ACN(aq) with 10% HCOOH. After evaporation, samples were reconstituted with 100 μL of 10% ACN(aq) with 0.1% HCOOH and filtered.

15193. CLSI guidelines were consulted for the design of the validation together with the validation approaches of other RMP methods (26–29). QCs from lowest to highest concentration (V1–V5) were prepared and analyzed in quintuplicate during 5 days for the evaluation of the method's precision, accuracy, and measurement uncertainty (MU).

SELECTIVITY

The chromatographic resolution of 1-84 PTH from other modified higher concentration PTH forms, such as oxidized PTH (preparation described in the Supplemental Material file) and 7-84 PTH, was tested. Ion ratios were compared between calibrators, QCs, and bona fide plasma samples (Supplemental Table 4). A difference of <20% was considered acceptable. The contribution of a blank matrix to the analyte and SIL-IS transitions, as well as the contribution of the IS to the analyte transition, are described in the Supplemental Material file.

Additionally, according to CLSI EP07 (30), the method was tested for interferences in samples with various degrees of hemolysis, lipemia, icterus, and hyperalbuminemia (Supplemental Material file).

LINEARITY

The linearity of the method was assessed according to the Study Design A1 described in CLSI guideline EP06 (31). As recommended, a human EDTA pool containing a high concentration of PTH was proportionally mixed with a blank sample (horse EDTA plasma) to constitute 9 relative concentrations at 1, 0.8, 0.5, 0.3, 0.15, 0.08, 0.04, 0.02, and 0.005, as shown in Supplemental Fig. 4, ranging from 5.6 to 1128.1 pg/L. Samples were prepared in quintuplicate and analyzed in the same run. The allowable deviation from linearity was defined at ±5% of the predicted values.

SAMPLE PREPARATION RECOVERY AND MATRIX EFFECTS

Absolute recovery and matrix effects were calculated at 25.0 pg/mL, 250.0 pg/mL, and 750.0 pg/mL, following a published procedure (32). PTH 1-84 standard stock solutions were prepared at 3 different concentrations (1.25 ng/mL, 12.5 ng/mL, and 37.5 ng/mL) and spiked in 3 sets of 1 mL samples in triplicate. Set A contained 80 µL of 10% ACN with 0.1% HCOOH. Sets B and C contained 1 mL of freshly pooled horse plasma.

Twenty microliters of stock solution were spiked into sets A and C. Sets B and C underwent sample preparation. After evaporation, Set B was reconstituted with 80 µL (10% ACN, 0.1% HCOOH) and spiked with 20 µL of the respective stock solution before the last filtering step.

Recovery and matrix effects were calculated according to the following equations:

$$Recovery\ (\%) = \frac{\mathrm{AUC(C)}}{\mathrm{AUC(B)}} \times 100$$

$$Matrix\ Effect(\%) = \frac{AUC(B)}{AUC(A)} \times 100$$

where the area under the ROC was the peak area for each set.

Additionally, the ion suppression through different matrices was assessed (Supplemental Material file).

CARRYOVER

Carryover was evaluated by injecting a sample containing 5000.0 pg/mL PTH (5× more concentrated than the highest calibration point) followed by a blank sample. Additionally, during the validation, samples were injected in the concentration order recommended by CLSI C64 section 8.8 (Carryover) (33) (i.e., medium [V3], high [V5], low [V1], medium, medium, low, low, high, high, medium), followed by the remaining validation samples injected in increasing levels of concentrations.

LOWER LIMIT OF THE MEASURING INTERVAL

The lower limit of the measuring interval (LLMI) was determined by the precision obtained from repetitions of the QC at 5.7 pg/mL. A CV < 20% was considered acceptable at the LLMI.

PRECISION AND ACCURACY

Precision and accuracy were evaluated by measuring 5 levels of spiked PTH-blank human EDTA plasma, in quintuplicate, during 5 days. Each day of validation, 5 aliquots per level were thawed and processed following the previously described procedure. Accuracy and precision for each QC level were estimated by single-nested ANOVA according to CLSI EP05 (34). Precision was expressed in terms of intra- and interday CV (%), calculated by single-nested ANOVA of daily means. The accuracy was expressed in terms of mean recoveries (%).

MEASUREMENT UNCERTAINTY ASSESSMENT

The APS defining the total budget for MU (TB_{mu}) were calculated according to the Joint Committee on Traceability in Laboratory Medicine Task Force on Reference Measurement System Implementation (35, 36). APS for reference methods are classified into 3 levels after PTH intrasubject biological variation expressed as CV_I %: minimum: $0.375 \times CV_I$; desirable $0.25 \times CV_I$; and optimal $0.125 \times CV_I$ (37). Following a top-down estimation of the MU or mu_{result} as recommended in ISO technical specification 20914 (38), the

contributions of the different sources of MU were defined along the traceability chain. Since a higher-order reference material does not exist for PTH, the standard value uncertainty ($mu_{value\ assignment}$) was obtained after amino acid analysis for the 1-84 PTH content based on the WHO 95/646 international standard performed by 5 independent laboratories (39). The attributable uncertainty of the calibrator (mu_{cal}) was defined as follows:

$$mu_{cal} = \sqrt{(mu_{value\ assignment})^2 + (mu_{dilution})^2 + (mu_{matrix})^2 + (mu_{IS})^2}$$

where $mu_{dilution}$ represents the uncertainty attributed to the preparation of the spiking solutions, and mu_{matrix} and mu_{IS} represent the uncertainty added through the pipetting of the IS and the matrix where the calibrators were prepared, respectively.

The mu_{result} (MU) was defined as:

$$mu_{result} = \sqrt{(mu_{cal})^2 + (mu_{rw})^2}$$

where mu_{rw} reflected the random variability of the measuring system, derived from the SD of the mean (single-nested ANOVA) obtained during the precision pool replicate studies, for each concentration.

The APS for an RMP was achieved if MU was equal to or less than the defined TB_{mu}. It has been recommended (36) that mu_{cal} should consume ≤ 50% TB_{mu}, leaving the remaining MU available for mu_{rw}.

METHOD COMPARISON

Different cohorts of samples and external QCs (EQC) were used to compare the results obtained with the LC-MS/MS and the third-generation PTH immunoassays from DiaSorin (Liaison XL) and Fujirebio Lumipulse.

The cohorts of samples comprised remnant EDTA plasma samples from 43 subjects presenting with an estimated glomerular filtration rate >60 mL/min/1.73 m^2 (non-CKD), 48 remnant EDTA samples from patients suffering from CKD, and 33 remnant samples obtained from patients on DIA.

The EQC samples (n = 35) from 2019, 2020, and 2021 were kindly supplied by UK NEQAS. These EQCs came as lyophilized tablets obtained from an EDTA pool with or without the addition of exogenous PTH. All samples were analyzed simultaneously with the 3 methods. Concordance correlation coefficient for the different compared groups was assessed and described in the Supplemental Material file.

STATISTICAL ANALYSIS

Passing–Bablok statistical regression and Bland–Altman analysis were used to determine proportional and systematic differences as well as linear deviations. EQCs were treated independently as they may not reflect a true human matrix.

Analyses of the method validation and comparison were performed using MedCalc version 20.009, R version 4.0.2, and MS Excel 2019. Analyst® Software v1.6.2 (Sciex) was used for system operation, and Multiquant® v3.0.8664 (Sciex) served for peak review and integration.

Results

METHOD SELECTIVITY

Three different forms of oxidized PTH (oxidation on methionine 8, methionine 18, and on both) and 7-84 PTH were successfully resolved by our liquid chromatography method (Supplemental Fig. 2). The mean ion ratios between the quantifier and qualifier transitions for native and internal standard were 2.24 ± 0.14.

LINEARITY

All 9 relative concentration levels were within allowable deviation from linearity with deviations ranging from −2.2% to 3.5%. The linearity profile is displayed in Supplemental Fig. 5.

SAMPLE PREPARATION RECOVERY AND MATRIX EFFECTS

Among the 3 tested concentrations, the absolute sample recoveries ranged from 34% to 41%. The calculated matrix effect spanned from 80% to 112%.

CARRYOVER

No deviation from noise was observed following a 5000.0 pg/mL injection (Supplemental Figs. 16 and 17). Carryover was thus considered negligible given the method's LLMI. QC levels during the method validation were not affected by the injection order.

LOWER LIMIT OF THE MEASURING INTERVAL

The QC level at 5.7 pg/mL showed a mean Signal-to-Noise (S/N) of 10:1 with a mean intraday CV of 3.0% and was thus defined as the LLMI. Supplemental Fig. 8 illustrates the limit of quantification response against a blank horse plasma sample.

PRECISION AND ACCURACY

In the validation, the intra- and interday imprecisions ranged from 0.4% to 5.4% and from 1.2% to 3.9%, respectively. Accuracy mean recoveries ranged from 96.2% to 103.2% (Table 1).

MEASUREMENT UNCERTAINTY ASSESSMENT

The European Federation of Clinical Chemistry and Laboratory Medicine has documented a CV_I value for PTH of 15.7% (40); therefore, the minimum, desirable,

Table 1. Precision and accuracy of the LC–MS/MS PTH measurements on plasma.

Sample	Concentration (pg/mL)	Day	Mean (pg/mL)	Recovery (%)	Intraday (CV,%)	Interday (CV,%)
1	872.6	1	864.7	99.1	1.4	3.9
		2	887.3	101.7	1.2	
		3	869.6	99.7	1.8	
		4	858.2	98.3	0.4	
		5	894.5	102.5	0.4	
2	484.8	1	481.2	99.3	1.1	1.7
		2	487.9	100.7	0.5	
		3	478.4	98.7	1.2	
		4	479.3	98.9	0.5	
		5	477.5	98.5	1.4	
3	47.9	1	47.5	99.2	0.8	1.2
		2	48.3	100.7	0.9	
		3	47.6	99.4	1.5	
		4	47.5	99.2	0.8	
		5	48.0	100.2	0.9	
4	9.7	1	9.4	97.4	1.9	1.3
		2	9.8	101.3	1.4	
		3	9.7	100.3	1.1	
		4	9.7	100.3	1.3	
		5	10.0	103.2	2.5	
5	5.7	1	5.5	96.2	5.4	2.1
		2	5.6	97.9	2.1	
		3	5.7	99.6	3.5	
		4	5.5	96.2	2.1	
		5	5.8	101.2	1.9	

and optimal APS for a reference PTH measurement are 5.9%, 3.9%, and 2.0%, respectively. Considering a $mu_{value\ assignment}$, $mu_{dilution}$, mu_{matrix}, and mu_{IS} of 3.6%, 0.4%, 1.6%, and 0.4%, respectively, the mu_{cal} was established at 4.0%. Values for mu_{rw} ranged from 1.2% to 3.9% for the different tested concentrations, which provided a MU that spanned from 4.2% to 5.6%, thus satisfying the TB_{mu} requirements for a RMP. The contribution of mu_{cal} to the TB_{mu} was above the recommended maximum uncertainty budget allowance (≤50%). The calculated measurement uncertainties are shown in Table 2.

METHOD COMPARISON

The previously described samples (n = 157), from normal estimated glomerular filtration rate, CKD, subjects on dialysis, and EQCs, were analyzed by the 3 systems. The obtained data were used to perform Passing–Bablok regressions and a Bland–Altman analysis (Figs. 2 and 3; Supplemental Figs. 9–12). In contrast to Fujirebio Lumipulse, the Liaison showed a higher proportional bias when compared to LC-MS/MS. Samples from patients undergoing DIA showed a seemingly higher degree of scatter by both immunoassays.

Representative patient chromatograms at 32.0 pg/mL and 234.0 pg/mL and an EQC at 87.0 pg/mL are displayed in Supplemental Figs. 13–15.

Discussion

Parathyroid hormone is routinely used for the exploration and management of different diseases, such as primary and secondary hyperparathyroidism or CKD–mineral bone disorder. Unfortunately, this test suffers a lack of standardization, which could lead to erroneous interpretation of the results. The standardization of PTH assays has thus become one of the priorities of the International Osteoporosis Foundation–IFCC for Bone Metabolism, but achieving this ambitious goal

Table 2. Estimation of the measurement uncertainties for LC–MS/MS PTH measurement.

	Level				
	1	2	3	4	5
Mean pool PTH concentration, pg/mL	5.7	9.7	47.9	484.8	872.6
SD of the mean (ANOVA), pg/mL	0.22	0.17	0.56	6.28	18.31
CV (mu_{rw}), %	3.9	1.7	1.2	1.3	2.1
Standard uncertainty ($mu_{value\ assignment}$), %			3.7		
Spiking solutions uncertainty ($mu_{dilution}$), %			0.4		
Calibration solutions uncertainty (mu_{matrix}), %			1.6		
Internal standard uncertainty (mu_{IS}), %			0.4		
Calibrator uncertainty (mu_{cal}), %			3.6		
Measurement uncertainty (mu_{result}), %	5.6	4.4	4.2	4.3	4.6

requires (a) a clear definition of the measurand, (b) a commutable internationally recognized international standard or a panel of samples with assigned values with an RMP, (c) at least one reference method procedure, and (d) international proficiency testing using values assigned with the RMP(s). Regarding the first prerequisite, there is little doubt that the most important analyte, responsible for the biological PTH activity, is the full sequence unmodified PTH form, which is the measurand of interest. Other fragments or forms have been described, but their use in daily practice remains to be clarified. Similar to another study (23), we could not detect any oxidized form of PTH nor 7-84 PTH in the samples we processed by monitoring their respective specific transitions. Regarding the second prerequisite, WHO 95/646 has been proposed as the international reference material, but matrix-based materials enriched with this standard have yet to be proven commutable. For the third prerequisite, so far there is no candidate RMP described for 1-84 PTH measurement. Finally, for the fourth prerequisite, an RMP for PTH, is also mandatory to precisely assign the concentrations of PTH in potential proficiency testing schemes. An RMP for PTH is urgently needed to move forward toward PTH standardization.

The method we describe allows the quantification of the intact 1-84 protein, avoiding tryptic digestion or immunocapture in the sample preparation, and responds to the analytical needs expressed by the IFCC Committee on Bone Metabolism for a higher-order method (41). The high precision, accuracy, and sensitivity achieved allow for the robust coverage of a wide range of PTH concentrations, as those observed in primary and secondary hyperparathyroidism as well as hypoparathyroidism.

The analysis of 1-84 PTH by LC-MS/MS poses a major challenge, given the complexity of liquid chromatography separation and electrospray ionization. An example of the latter was observed during the fragmentation of 1-84 PTH by collision induced fragmentation. For multiple reaction monitoring quantitation, only the y82 fragment provided a sufficiently sensitive transition, illustrating a clear technical limitation that prevented the obtention of independent multiple reaction monitoring transitions. In addition, the high value and the width of the charge state envelope can be sources of lack of analytical selectivity and sensitivity in a tandem quadrupole mass spectrometer. The combination of a rather selective and enriching SPE, with a well-performing liquid chromatography, were critical to overcome these difficulties and to achieve satisfactory APS.

Even if the contribution of the calibrators to the TB_{mu} was above the recommended 50%, it was deemed suitable for the purpose of this work. Unfortunately, the calibration used in this method employed a standard whose concentration was assigned using an amino acid analysis without a well-characterized purity value and lacking a state-of-the-art uncertainty assessment, which are necessary for a primary calibrator in an RMP. A new primary reference material, with better characterized purity and traceable to the SI units, would be necessary to adapt our work to become a candidate RMP.

Interestingly, we compared the results we obtained with the newly developed method and third-generation PTH assays (DiaSorin Liaison and Fujirebio Lumipulse), notably in patients with CKD and patients on DIA. The results showed a moderate to substantial correlation between the 3 methods despite a substantial proportional bias observed with DiaSorin. This is likely attributable to the different standards used to calibrate the methods (WHO 95/646 and Bachem, respectively), whereas Fujirebio Lumipulse was calibrated with the same WHO 95/646 standard. It would be of value to compare the results obtained with different second-generation PTH

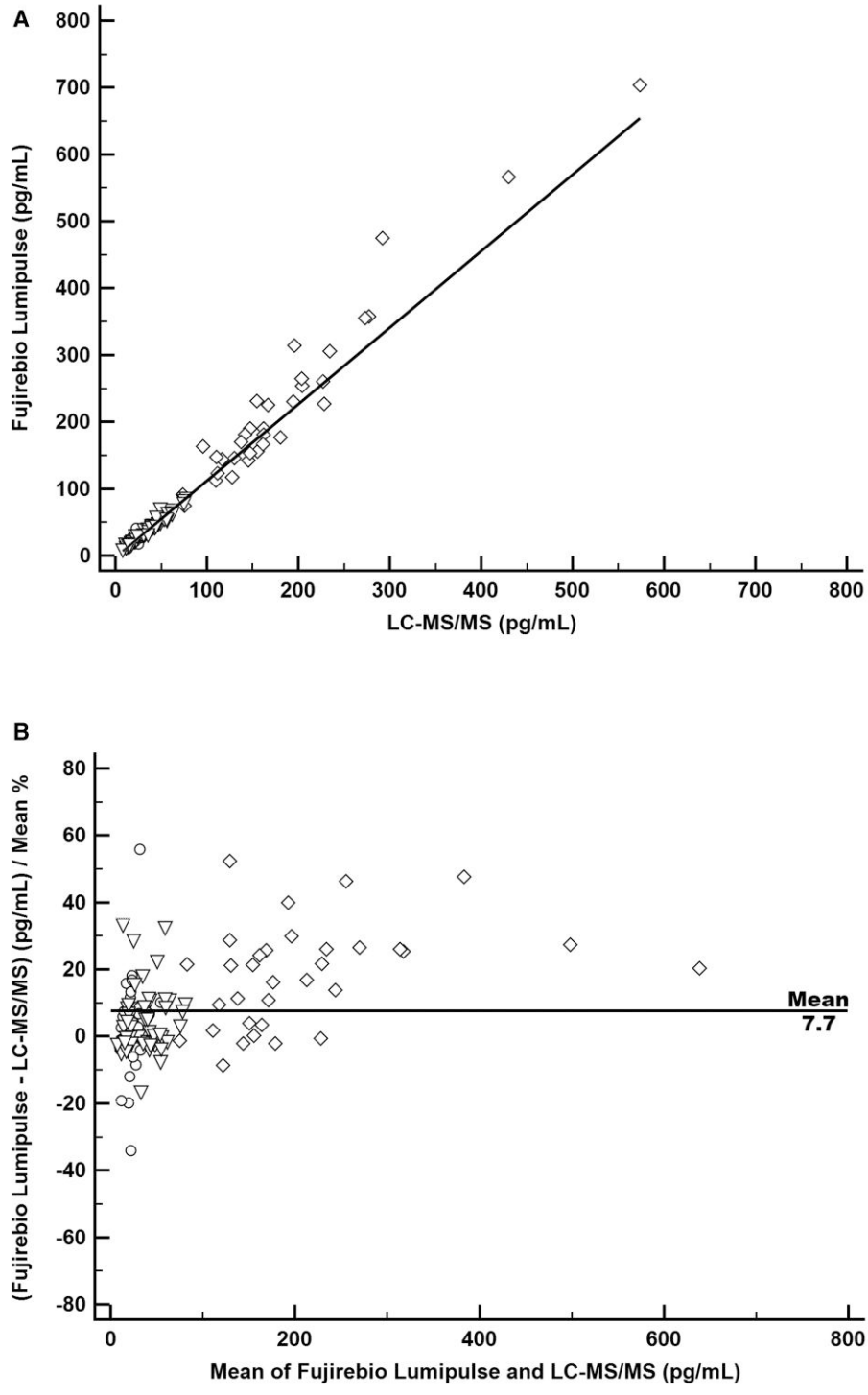
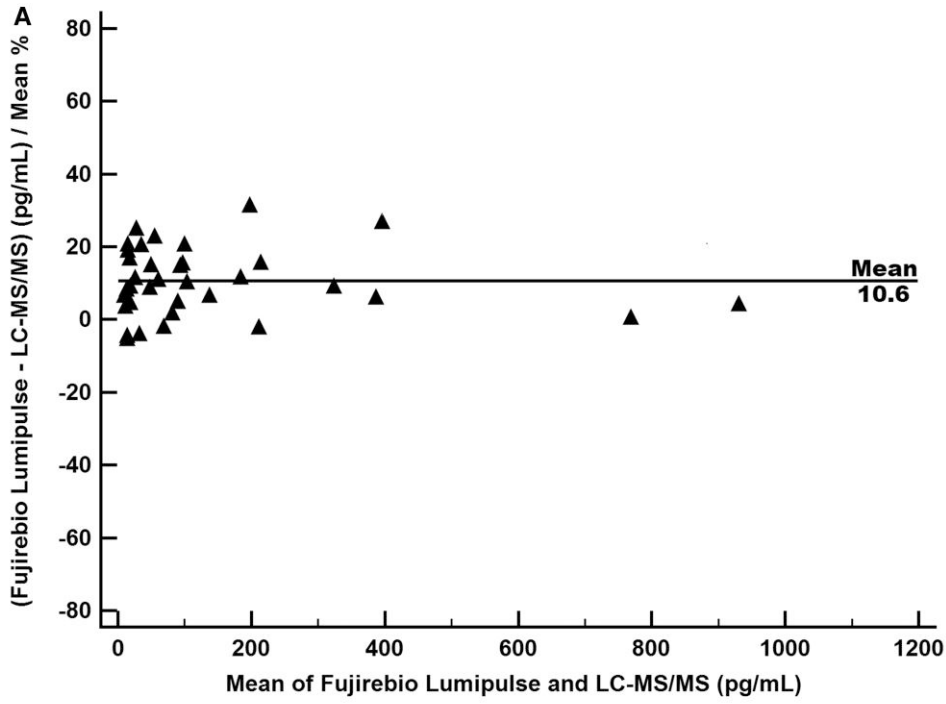


Fig. 2. (A), Fujirebio Passing–Bablok regression and Bland–Altman plots for non-CKD, CKD, and DIA (n = 124). Intercept pg/mL (95% CI): −2.35 (−4.08 to −1.07). Slope (95% CI): 1.14 (1.09 to 1.22). $R^2 = 0.975$; (B), Bland–Altman plot for non-CKD, CKD, and DIA. Mean of the difference from the 2 measurements is represented by the solid line at 7.7%. Circles and inverted triangles represent non-CKD and CKD samples respectively. Diamonds represent DIA samples.



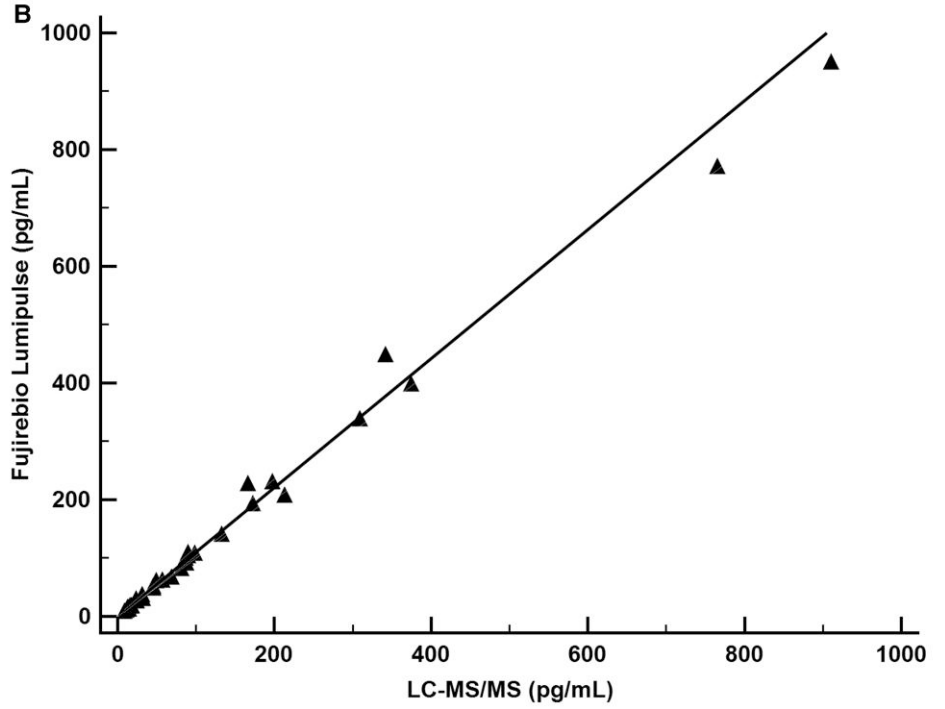


Fig. 3. (A), Fujirebio Passing–Bablok regression for the EQCs (n = 35). Intercept pg/mL (95% CI): −0.15 (−1.80 to 1.95). Slope (95% CI): 1.11 (1.05 to 1.18). $R^2 = 0.996$; (B), Bland–Altman plot for the EQCs. Mean of the difference from the 2 measurements is represented by the solid line at 10.6%.

assays to investigate whether they could be standardized and/or harmonized in patients with and without CKD. Indeed, in patients with CKD and patients on DIA, the accumulation of fragments is known to overestimate PTH concentrations, even if a recent study did not find any interference of the circulating fragments with the Roche second-generation PTH assay (23).

In conclusion, we believe that the presented work is an important but preliminary step to an RMP, standing by for a higher-order reference standard, with state-of-the-art metrological purity assessment and uncertainty evaluation. Additionally, multicenter and multiethnic reference ranges should be established using an appropriate methodology. Harmonization and/or standardization of the different kits available on the market should be achieved and monitored over time. Once all the steps are accomplished, this could improve the situation that clinicians and laboratories face today regarding the lack of standardization of PTH assays.

Supplementary Material

Supplementary material is available at *Clinical Chemistry* online.

Nonstandard Abbreviations: PTH, parathyroid hormone; CKD, chronic kidney disease; DIA, hemodialysis; RMP, reference measurement procedures; APS, analytical performance specifications; ACN, acetonitrile; SPE, solid-phase extraction; SIL-IS, stable isotope-labeled internal standard; QC, quality control; MU, measurement uncertainty; LLMI, lower limit of the measuring interval; TB_{mu}, total budget for MU; EQC, external quality control.

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References

1. Pfister MF, Ruf I, Stange G, Ziegler U, Lederer E, Biber J, Murer H. Parathyroid hormone leads to the lysosomal degradation of the renal type II Na/Pi cotransporter. Proc Natl Acad Sci U S A 1998;95:1909–14.
2. Fermor B, Skerry TM. PTH/PTHrP receptor expression on osteoblasts and osteocytes but not resorbing bone surfaces in growing rats. J Bone Miner Res 1995;10:1935–43.
3. Leiker AJ, Yen TWF, Eastwood DC, Doffek KM, Szabo A, Evans DB, Wang TS. Factors that influence parathyroid hormone half-life: determining if new intraoperative criteria are needed. JAMA Surg 2013;148:602–6.
4. Segre G V, Perkins AS, Witters LA, Potts JT. Metabolism of parathyroid hormone by isolated rat Kupffer cells and hepatocytes. J Clin Invest 1981;67:449–57.
5. Hanley DA, Ayer LM. Calcium-dependent release of carboxyl-terminal fragments of parathyroid hormone by hyperplastic human parathyroid tissue in vitro. J Clin Endocrinol Metab 1986;63:1075–9.
6. Mayer GP, Keaton JA, Hurst JG, Habener JF. Effects of plasma calcium concentration on the relative proportion of hormone and carboxyl fragments in parathyroid venous blood. Endocrinology 1979;104:1778–84.
7. D'Amour P, Lazure C, France L. Metabolism of radioiodinated carboxy-terminal fragments of bovine parathyroid hormone in normal and anephric rats. Endocrinology 1985;117:127–34.
8. D'Amour P. Circulating PTH molecular forms: what we know and what we don't. Kidney Int 2006;70:S29–33.
9. Costa AG, Cremers S, Dworakowski E, Lazaretti-Castro M, Bilezikian JP. Comparison of two commercially available ELISAs for circulating sclerostin. Osteoporos Int 2014;25:1547–54.
10. Nussbaum SR, Zahradnik RJ, Lavigne JR, Brennan GL, Nozawa-Ung K, Kim LY, et al. Highly sensitive two-site immunoradiometric assay of parathyrin, and its clinical utility in evaluating patients with hypercalcemia. Clin Chem 1987;33:1364–7.
11. Lepage R, Roy L, Brossard JH, Rousseau L, Dorais C, Lazure C, D'Amour P. A non-(1-84) circulating parathyroid hormone (PTH) fragment interferes significantly with intact PTH commercial assay measurements in uremic samples. Clin Chem 1998;44:805–9.
12. Quarles LD, Lobaugh B, Murphy G. Intact parathyroid hormone overestimates the presence and severity of parathyroid-mediated osseous abnormalities in uremia. J Clin Endocrinol Metab 1992;75:145–50.
13. Wang M, Hercz G, Sherrard DJ, Maloney NA, Segre G V, Pei Y. Relationship between intact 1-84 parathyroid hormone and bone histomorphometric parameters in dialysis patients without aluminum toxicity. Am J Kidney Dis 1995;26:836–44.
14. John MR, Goodman WG, Ping G, Cantor T, Salusky IB, Juppner H. A novel immunoradiometric assay detects full-length human PTH but not amino-terminally truncated fragments: implications for PTH measurements in renal failure. J Clin Endocrinol Metab 1999;84:4287–90.
15. Gao P, Scheibel S, D'Amour P, John MR, Rao SD, Schmidt-Gayk H, Cantor TL. Development of a novel immunoradiometric assay exclusively for biologically active whole parathyroid hormone 1-84: implications for improvement of accurate assessment of parathyroid function. J Bone Miner Res 2001;16:605–14.
16. Ursem SR, Heijboer AC, D'Haese PC, Behets GJ, Cavalier E, Vervloet MG, Evenepoel P. Non-oxidized parathyroid

hormone (PTH) measured by current method is not superior to total PTH in assessing bone turnover in chronic kidney disease. Kidney Int. 2021;99:1173–8.
17. D'Amour P, Brossard JH, Rousseau L, Roy L, Gao P, Cantor T. Amino-terminal form of parathyroid hormone (PTH) with immunologic similarities to hPTH(1-84) is overproduced in primary and secondary hyperparathyroidism. Clin Chem 2003;49:2037–44.
18. Cavalier E, Daly AF, Betea D, Pruteanu-Apetrii PN, Delanaye P, Stubbs P, et al. The ratio of parathyroid hormone as measured by third- and second-generation assays as a marker for parathyroid carcinoma. J Clin Endocrinol Metab 2010;95:3745–9.
19. Cavalier E, Betea D, Schleck ML, Gadisseur R, Vroonen L, Delanaye P, et al. The third/second generation PTH assay ratio as a marker for parathyroid carcinoma: evaluation using an automated platform. J Clin Endocrinol Metab 2014;99:453–7.
20. Arakawa T, D'Amour P, Rousseau L, Brossard JH, Sakai M, Kasumoto H, et al. Overproduction and secretion of a novel amino-terminal form of parathyroid hormone from a severe type of parathyroid hyperplasia in uremia. Clin J Am Soc Nephrol 2006;1:525–31.
21. Kumar V, Barnidge DR, Chen LS, Twentyman JM, Cradic KW, Grebe SK, Singh RJ. Quantification of serum 1-84 parathyroid hormone in patients with hyperparathyroidism by immunocapture in situ digestion liquid chromatography-tandem mass spectrometry. Clin Chem 2010;56:306–13.
22. Lopez MF, Rezai T, Sarracino DA, Prakash A, Krastins B, Athanas M, et al. Selected reaction monitoring-mass spectrometric immunoassay responsive to parathyroid hormone and related variants. Clin Chem 2010;56:281–90.
23. Kritmetapak K, Losbanos LA, Hines JM, O'Grady KL, Ulmer CZ, Vesper HW, et al. Chemical characterization and quantification of circulating intact PTH and PTH fragments by high-resolution mass spectrometry in chronic renal failure. Clin Chem 2021;67:843–53.
24. Cavalier E, Delanaye P, Vranken L, Bekaert AC, Carlisi A, Chapelle JP, Souberbielle J-C. Interpretation of serum PTH concentrations with different kits in dialysis patients according to the KDIGO guidelines: importance of the reference (normal) values. Nephrol Dial Transplant 2012;27:1950–6.
25. Cavalier E, Salsé M, Dupuy AM, Bargnoux AS, Watar F, Souberbielle JC, et al. Establishment of reference values in a healthy population and interpretation of serum PTH concentrations in hemodialyzed patients according to the KDIGO guidelines using the lumipulse® G whole PTH (3rd generation) assay. Clin Biochem 2018;54:119–22.
26. Tai SSC, Bedner M, Phinney KW. Development of a candidate reference measurement procedure for the determination of 25-hydroxyvitamin D3 and 25-hydroxyvitamin D 2 in human serum using isotope-dilution liquid chromatography tandem mass spectrometry. Anal Chem 2010;82:1942–8.
27. Tai SSC, Nelson MA. Candidate reference measurement procedure for the determination of (24 R),25-dihydroxyvitamin D3 in human serum using isotope-dilution liquid chromatography-tandem mass spectrometry. Anal Chem 2015;87:7964–70.
28. Leinenbach A, Pannee J, Dülffer T, Huber A, Bittner T, Andreasson U, et al. Mass spectrometry-based candidate reference measurement procedure for quantification of amyloid-β in cerebrospinal fluid. Clin Chem 2014;60:987–94.
29. Marcovina SM, Clouet-Foraison N, Koschinsky ML, Lowenthal MS, Orquillas A, Boffa MB, et al. Development of an LC-MS/MS proposed candidate reference method for the standardization of analytical methods to measure lipoprotein(a). Clin Chem 2021;67:490–9.
30. CLSI. Clinical and Laboratory Standards Institute guideline EP07: interference testing in clinical chemistry, 3rd Ed. Wayne (PA): CLSI; 2021.
31. CLSI. Clinical and Laboratory Standards Institute guideline EP06: evaluation of linearity of quantitative measurement procedures. Wayne (PA): CLSI; 2020.
32. Matuszewski BK, Constanzer ML, Chavez-Eng CM. Strategies for the assessment of matrix effect in quantitative bioanalytical methods based on HPLC–MS/MS. Anal Chem 2003;75:3019–30.
33. Clinical and Laboratory Standards Institute (CLSI) guideline C64: quantitative measurement of proteins and peptides by mass spectrometry, 1st ed. Wayne, PA: Clinical and Laboratory Standards Institute; 2021.
34. Durham AP, Goldford MD, Kondratoovich M V, Middle JG, Vaks JE. CLSI EP05-A3: 2014 evaluation of precision of quantitative measurement procedures; approved guideline, 3rd ed. Wayne, PA: Clinical and Laboratory Standards Institute; 2022.
35. Panteghini M, Braga F. Implementation of metrological traceability in laboratory medicine: where we are and what is missing. Clin Chem Lab Med 2020;58:1200–4.
36. Braga F, Panteghini M. The utility of measurement uncertainty in medical laboratories. Clin Chem Lab Med 2020;58:1407–13.
37. Hawley JM, Owen LJ, Mackenzie F, Mussell C, Cowen S, Keevil BG. Candidate reference measurement procedure for the quantification of total serum cortisol with LC-MS/MS. Clin Chem 2016;62:262–9.
38. ISO/TS. 20914:2019 Medical laboratories—practical guidance for the estimation of measurement uncertainty. 1st Ed. Geneva (Switzerland): International Organization for Standardization (ISO); 2019.
39. Burns C, Ellis M, Hockley J, Rigsby P; World Health Organization, Biologicals Unit. WHO international collaborative study of the proposed 1st [first] international standard for parathyroid hormone 1-84, human, recombinant. Geneva (Switzerland): WHO; 2009.
40. Aarsand AK, Fernandez-Calle P, Webster C, Coskun A, Gonzales-Lao E, Diaz-Garzon J, et al. The EFLM Biological Variation Database. https://biologicalvariation.eu/ (Accessed October 2021).
41. Sturgeon CM, Sprague S, Almond A, Cavalier E, Fraser WD, Algeciras-Schimnich A, et al. Perspective and priorities for improvement of parathyroid hormone (PTH) measurement—a view from the IFCC working group for PTH. Clin Chim Acta 2017;467:42–7.