Quantification of Serum 1–84 Parathyroid Hormone in Patients with Hyperparathyroidism by Immunocapture In Situ Digestion Liquid Chromatography–Tandem Mass Spectrometry

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BACKGROUND: Immunoassays specific for 1–84 parathyroid hormone (PTH) reportedly reflect the bioactivity of PTH; however, PTH immunoassays can be susceptible to interference by cross-reacting PTH fragments. In addition, these assays currently lack standardization. A methodology using immunocapture purification with liquid chromatography–tandem mass spectrometry (LC-MS/MS) detection, along with a stable isotope–labeled internal standard, may help address these issues.

METHODS: We isolated 1–84 PTH from 1 mL serum by immunocapture on a 6.5-mm polystyrene bead. The immobilized PTH was digested in situ and analyzed by LC-MS/MS. For quantification, we used the selected reaction monitoring response from the N-terminal tryptic peptide 1–13 PTH (1SVSEIQLMHNLGK13).

RESULTS: The linear range of the assay was 39.1–4560 ng/L, and the limit of detection and limit of quantification were 14.5 ng/L and 39.1 ng/L, respectively. The intraassay CVs ranged from 6% to 11%, and the interassay CVs ranged from 7% to 17%. Interference by PTH fragments 1–44 PTH, 7–84 PTH, 43–68 PTH, 52–84 PTH, 64–84 PTH, and PTH-related protein (PTHrP) was 0% to 0.001%. Method comparison of LC-MS/MS vs the Roche Cobas® immunoassay yielded Deming fit of LC-MS/MS vs immunoassay = 1.01 x immunoassay – 13.21. The mean bias by Bland–Altman plot was –9.4%.

CONCLUSIONS: In patients with hyperparathyroidism, the immunocapture in situ digestion LC-MS/MS method can provide accurate and precise PTH results compared with immunoassay.

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The pivotal role of parathyroid hormone (PTH)2 in calcium regulation makes its measurement an important part of the assessment of hypocalcemia, hypercalcemia, metabolic bone disease, and parathyroid gland tumors. PTH is synthesized in the parathyroid glands as a 115–amino acid precursor (prepro-PTH), cleaved to pro-PTH, and then to the bioactive 84–amino acid polypeptide (1–84 PTH or intact PTH). After secretion, 1–84 PTH has been reported to undergo rapid metabolism to form carboxyl-terminal, amino-terminal, and midmolecule fragments (1, 2). With mass spectrometry–based methods, 4 human C-terminal PTH peptide fragments, hPTH(34–84), hPTH(37–84), hPTH(38–84), and hPTH(45–84), have been identified from plasma of healthy postmenopausal women who were given 100 μg recombinant hPTH by subcutaneous injection or a 15-min intravenous infusion (1). Similar fragments were also observed in a plasma pool from patients with chronic renal insufficiency (1). In a different study, additional C-terminal PTH fragments were also identified from parathyroid cells isolated from glands obtained at surgery from 3 patients with primary hyperparathyroidism and 3 patients with secondary hyperparathyroidism, after these cells were incubated with 35S-methionine to internally label their secretion products (2). PTH polypeptides containing the first 3–6 N-terminal amino acids of 1–84 PTH can activate the parathyroid hormone receptor type 1 (PTH-R1) and have half-lives of approximately 5 min in circulation. Inactive PTH fragments, on the other hand, have half-lives of 24–36 h and make up >90% of total circulating PTH (3). C-terminal fragments in particular can accumulate to very high concentrations in renal failure because they are primarily cleared by the kidneys (3).
Consequently, the ability of any PTH assay to accurately diagnose hyper- or hyposecretion of 1–84 PTH is closely related to its degree of cross-reactivity with the midmolecule and C-terminal cleavage fragments. First-generation PTH immunoassays displayed substantial cross-reactivity with various PTH fragments. Second-generation PTH immunoassays in sandwich format replaced the original assays during the last 2 decades and have performed substantially better than older assays. While they were designed to detect intact 1–84 PTH, they have been shown to cross-react with long C-terminal PTH fragments, chiefly 7–84 PTH (4). These fragments do not stimulate the PTHR-1, but, depending on the second-generation assay used, they account for 10%–30% of PTH serum immunoreactivity in patients with normal renal function, and ≥45% in renal failure (3). Therefore, as measures of 1–84 PTH, even second-generation PTH assays perform suboptimally in renal failure. In addition, it has become apparent that high concentrations of 7–84 PTH and some other C-terminal PTH fragments may oppose the biochemical and bone-metabolic effects of 1–84 PTH (5–7), aggravating the potential undesirable clinical consequences of overestimating 1–84 PTH concentrations in renal failure patients.

To address these problems, third-generation PTH immunoassays have been developed with higher specificity for 1–84 PTH (8, 9). In patients with normal renal function, these assays perform at least as well as second-generation assays, with some evidence for superior performance in the diagnosis of primary hyperparathyroidism (10). However, the main strength of these new assays lies in PTH measurement in renal failure patients, as these assays allow unequivocal measurement of “true” 1–84 PTH. Unfortunately, some third-generation PTH immunoassays have shown problems with calibration drift and were recently discontinued (11).

Our laboratory addressed similar issues related to immunoassays for steroids by implementing liquid chromatography–tandem mass spectrometry (LC-MS/MS) methods that are now used in clinical laboratories (12). For quantification of full-length 1–84 PTH by LC-MS/MS, our initial experiments with solid-phase extraction (SPE) purification lacked adequate signal to noise for the method to be clinically viable. We hypothesized that to increase the signal-to-noise ratio, we needed to immunocapture PTH from serum for enrichment, wash the bead to remove interferences and ion suppressing matrices, digest bound material with trypsin to generate the N-terminal 1–13 PTH tryptic peptide, and quantify 1–13 PTH by LC-MS/MS (Fig. 1). We describe here a validated immunocapture in situ digestion LC-MS/MS assay that has been shown to detect bioactive PTH containing the intact N-terminus in human serum.

Materials and Methods

REAGENTS

We obtained recombinant 1–84 PTH from the National Institute for Biological Standards and Controls (NIBSC), Hertfordshire, UK. Stock solutions of 1–84 PTH were prepared at a concentration of 1 g/L in HPLC-grade water containing 200 mmol/L formic acid (Fisher Scientific) and stored at −80 °C until use. To optimize the MS/MS and HPLC conditions, synthetic 1–13 PTH peptide (1SVSEIQLMHNLGK13) was made by the Mayo Clinic Rochester proteomics research peptide synthesis facility on an ACT 396 Multiple Peptide Synthesizer (Advanced ChemTech), using recommended procedures for 1,3-diisopropylcarbodiimide

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activation and coupling. The amino acid sequence of the synthetic peptide was confirmed by LC-MS/MS using a Waters nanoAcquity LC and a Waters Q-TOF Premier quadrupole-time-of-flight mass spectrometer (Waters Corp.). The purity of the synthetic peptide was found by amino acid analysis to be 89%. To achieve the appropriate precision in LC-MS/MS methods, we considered an internal standard essential. Because no stable isotopically labeled 1–84 PTH was commercially available, we synthesized 15N stable isotope–labeled 1–84 PTH (15NrPTH) in the laboratory. 15NrPTH was expressed in E. coli grown on OD-2N media containing 15N-labeled essential compounds (Silantes). Using the PET32a expression system (Novagen), we constructed a vector containing a PTH sequence in which the nucleic acid codon selection for the individual encoded amino acids had been modified to be optimized for efficient expression in E. coli and purified the expressed protein using the method described by Liu et al. (13). The purified 15NrPTH was analyzed by SDS-PAGE and estimated to be 90% pure by Coomassie staining. We also analyzed the protein by LC-MS/MS using the Q-TOF Premier and found it to have an intact mass near the calculated mass of 1–84 PTH, assuming 100% incorporation of the 15N stable isotope label. For interference studies, we purchased synthetic 7–84 PTH from Bachem in lyophilized form. We purchased other PTH fragments (1–44 PTH, 43–68 PTH, 52–84 PTH, 64–84 PTH) and parathyroid hormone–related protein (PTHrP), reported to be 85% pure, from Sigma.

For immunopurification and separation of PTH from human serum, we purchased polystyrene beads coated with murine monoclonal antibodies recognizing the 44–84 region of PTH from Siemens (Immulette™ 2000 PTH assay). We purchased second-generation automated PTH immunoassay reagents and equipment (Cobas) from Roche Diagnostics. The Roche Cobas PTH assay is a sandwich immunoassay that uses a capture antibody that recognizes the C-terminal 38–84 PTH epitope and a reporter antibody that binds to the N-terminal 1–37 PTH epitope, followed by electrochemiluminescence detection.

The 96–deep well polypropylene microtiter plates were from ChromTech Inc., the titer plate shaker from Lab-Line Instruments Inc., and the shaking incubator from Thermo Scientific. Trypsin and ammonium bicarbonate used for protein digestion were from Sigma.

CALIBRATOR PREPARATION AND PATIENT SAMPLE HANDLING
We prepared 12 calibrators, ranging in concentration from 20 to 4560 ng/L, by diluting a NIBSC 1–84 PTH standard material into charcoal-stripped human serum (SeraConII; SeraCare Diagnostics) and kept them frozen at −20 °C until needed. The recombinant stable isotope-labeled internal standard 15NrPTH was diluted into charcoal-stripped human serum at a concentration of 8 ng/L and frozen at −20 °C until needed. Patient samples used in this study were collected in plain red-top tubes, serum separating tubes, or EDTA tubes.

WORK FLOW OF SAMPLE PREPARATION
Fig. 1 details the immunocapture in situ digestion LC-MS/MS work flow that included immunocapture of PTH and spiked 15NrPTH internal standard from the sample, washing of the bead, in situ digestion with trypsin, and quantification of prototypic peptide by LC-MS/MS. We performed experiments to determine the optimum (1) incubation time (4 h), (2) number of washing steps (2 PBS washes), and (3) trypsin digestion time (30 min) (data not shown).

LC-MS/MS PARAMETERS
We analyzed all samples using a Thermo-Cohesive HPLC system (Thermo Scientific) coupled to an Applied Biosystems API 5000 triple-quadrupole mass spectrometer. After digestion, samples in the 96-well plate were loaded onto the CTC Analytics autosampler (LEAP Technologies) for LC-MS/MS analysis, and a 100-μL injection was made from each sample. Chromatographic separation was carried out on a 50- by 2.1-mm Targa C18 column with 3-μm particle size and 120-Å pore size (Higgins Analytical) at a flow rate of 250 μL/min. A gradient consisting of mobile phase A (100% water, 0.1% formic acid) and mobile phase B (100% methanol, 0.1% formic acid) was used to resolve the peptides. The gradient started at 20% mobile phase B and was held for 2 min before ramping to 35% B (4 min) → 42% B (6 min) → 95% B (2 min) → 20% B (1 min). The gradient was held at 20% mobile phase B for 5 min before the next injection.

All MS/MS conditions were optimized using the synthetic 1–13 PTH peptide by infusing a peptide solution through a “tee” into a 250-μL/min flow stream consisting of 40% mobile phase A. The transition that gave the best signal-to-noise ratio for 1–13 PTH was the triply charged precursor ion to the doubly charged product ion ([M+3H+]3+ → y11+2) translating to a selected reaction monitoring (SRM) transition of 486.2 m/z → 635.4 m/z. The corresponding SRM transition for isotope labeled 1–13 PTH internal standard was 492.2 m/z → 643.7 m/z. The API 5000 instrument parameters for tryptic peptide 1–13 PTH multiple reaction monitoring were as follows: CAD, 12; CUR, 45; GS1, 35; GS2, 30; IS, 5500; TEM, 600; DP, 120; EP, 10.8; CE, 26; CXP, 42. Analyst™ software version 1.4.2 (Applied Biosystems) was used to control the instrument and acquire and process the data.
ION SUPPRESSION STUDIES
We used a postcolumn infusion method to assess susceptibility of the method to ion suppression. Briefly, we connected a syringe pump via a tee to the column effluent and infused 1–13 PTH at a flow rate of 20 μL/min directly into the source of electrospray ionization MS/MS, resulting in a constant response (data not shown). A stripped serum blank and a pooled serum control that had undergone immunocapture followed by trypsin digestion were also injected into the column, and the effect of matrix suppression on the response for the continuously infused 1–13 PTH was monitored (14).

VALIDATION STUDIES
The mean molecular weight of 1–84 PTH is 9425 Da, and is converted to pmol/L by multiplying the ng/L values by 0.106. We determined the reproducibility of the standard curve by comparing the LC-MS/MS standard curves obtained over 10 different days (Fig. 2). We assessed intra- and interassay imprecision by analyzing 20 replicates each of 4 patient serum pools (39, 55, 145, and 300 ng/L) (Fig. 3).

We calculated the limit of detection (LOD) for 1–84 PTH in serum, defined as the concentration that gave the response greater than the response of stripped serum blank + 3 SDs, by analyzing stripped serum blanks and samples near the estimated limit of detection over 10 days. We determined the limit of quantitation (LOQ), defined as the lowest measurable analyte level with an interassay measurement CV of <20%, by analyzing repeatedly 2 replicates each of 5 pooled serum controls over a period of 10 days.

Because there is no established reference measurement procedure for quantifying 1–84 PTH, we performed a recovery study to assess the accuracy of quantification of 1–84 PTH by LC-MS/MS method. Samples at 3 different PTH concentrations [88.8, 179, and 461 ng/L (9.4, 19, and 49 pmol/L)] were spiked into 3 aliquots each of 3 different pooled serum control samples [1–84 PTH concentrations 75.2, 201, and 426 ng/L (8, 21, 45 pmol/L)] and analyzed by LC-MS/MS. The percent recovery was calculated from the measured vs expected PTH values (Table 1).

Dilution linearity was assessed using 3 pooled patients’ controls [1–84 PTH: 1430, 323, and 233 ng/L (152, 34 and 25 pmol/L)], which were diluted 1/2- to 1/32-fold in stripped serum. We then measured 1–84 PTH by the LC-MS/MS method, plotted the 1–84 PTH values obtained against the percent dilution of samples, and performed linear regression.

INTERFERENCE STUDIES
To further assess if the LC-MS/MS method measured 1–84 PTH accurately, we spiked a pooled patient serum sample (PTH value of 63.1 ng/L; 6.7 pmol/L) with PTHrP and a variety of N- and C-terminus PTH fragments between 500 and 5 000 000 ng/L and compared the measured values with the nonspiked sample. We defined “no interference” as results after spike being unchanged or within 10% of the original results.

To assess the effect of hemolysis, we prepared hemolysate from whole EDTA blood by washing the red blood cells with PBS, pH 7.4, twice, followed by freezing at −20 °C to lyse the red blood cells. We measured the hemoglobin concentration using HemoCue AB. We spiked 3 pooled patient controls (PTH: 50.1, 90.0, and 145.0 ng/L) with 0.1 mL of either PBS or hemolysate.
sate (hemoglobin 0.50–10 g/L) and analyzed the samples.

To determine if extremely high concentrations of lipids in a serum sample may interfere, we mixed 1 volume of 3 pooled patient samples with 3 volumes of a grossly lipemic pooled sample (lipemic index 4). We then quantified the concentration of 1–84 PTH both before and after ultracentrifugation to remove lipids.

We tested the effects of icteric samples on assay performance by mixing pooled patient samples [PTH values 61, 150, and 282 ng/L (6.5, 16 and 30 pmol/L)] in a ratio of 1–3 with an icteric sample (bilirubin value 28 mg/L). We quantified the 1–84 PTH in the neat controls, the icteric sample, and the samples mixed with icteric sample.

**METHOD COMPARISON**

We collected 141 serum samples with PTH concentrations between 40 and 737 ng/L measured with the Roche Cobas PTH assay. We also compared 15 samples with a third-generation PTH immunoassay from Scantibodies Laboratory. These samples were stored frozen at −20 °C until 1–84 PTH was quantified using the LC-MS/MS method. This study was approved by the Mayo Clinic Rochester Institutional Review Board.

**CALCULATIONS AND STATISTICS**

Data were analyzed using Microsoft Excel 2003, Analyse-it® (Analyse-it Software), or JMP (SAS).

**Results**

**PERFORMANCE CHARACTERISTICS OF IMMUNOCAPTURE IN SITU LC-MS/MS**

Calibration-standard concentrations for 1–84 PTH ranged from 20 to 4560 ng/L (2 to 483 pmol/L). The calibration curves were linear throughout this range and reproducible (r² = 0.997, intercept 0.646, slope 0.9992) (Fig. 2 and Supplemental Data 1, which accompanies the online version of this article at http://www.clinchem.org/content/vol56/issue2). The intra- and interassay measurements were used to determine the %CV of the imprecision, and data are presented in Supplemental Data 2 and Supplemental Fig. 1. Consistent with the signal to noise observed in typical chromatography, the LOD was determined to be 14.5 ng/L (1.5 pmol/L) and the LOQ to be 39.1 ng/L (4 pmol/L).

Fig. 3 shows the assay CVs vs PTH concentration. Representative chromatograms of blank serum, calibration standards, control material, and patient samples are shown in Fig. 4. The mean overall percentage recovery for spiked 1–84 PTH was 102.9% (range 89.3% to 117%) (Table 1).

When a high control sample was diluted with a low control, the expected PTH concentration was observed to be linear (r² ≥ 0.990) up to the LOQ (see online Supplemental Fig. 2). The LC-MS/MS method did not exhibit any ion suppression from the serum sample matrix; most of the suppressing compounds are apparently removed during the immunocapture purification step (data not shown).

**INTERFERENCE STUDIES**

Addition of 7–84 PTH up to a concentration of 5 μg/L did not interfere with the quantification of 1–84 PTH. The PTH fragments 52–84 PTH and 64–84 PTH also showed no interference when spiked into serum up to a concentration of 0.5 ng/L. PTH fragment 43–68 PTH did not show interference up to a concentration of 50 μg/L. 1–44 PTH did not alter the concentration of 1–84 PTH in a patient serum up to 5 μg/L. PTHrP spiked in patient serum up to 0.5 mg/L also did not interfere with the assay. In the hemolysis experiments, the recovery of 1–84 PTH from all the pooled patients’ controls was within 10% of expected concentrations (n = 15), indicating no significant interference from hemolysis at hemoglobin concentrations ≤10 g/L. For the lipemic sample dilutions, where the sample was...
first ultracentrifuged to remove lipid, the percent recovery of 1–84 PTH was within 10% \((n = 3)\), indicating the absence of interference. For the icteric sample dilutions, the samples were also found to be within the 10% limit \((n = 9)\), indicating that icterus did not interfere with PTH quantification by LC-MS/MS.

**METHOD COMPARISON**

We compared the Roche Cobas immunoassay values for 141 patient samples between 40 and 737 ng/L \((4 \text{ and } 78 \text{ pmol/L})\) with those obtained by the LC-MS/MS method. The weighted Deming fit plot (Fig. 5A) showed a slope of 1.01 and an intercept of \(-13.21\), with SD of residuals 0.203, consistent with the Bland–Altman analysis, which showed a slight overall negative bias of \(-9.4\%\) for the LC-MS/MS method compared with Roche Cobas (Fig. 5B). However, significant difference in PTH results was observed between the LC-MS/MS and the Scantibodies third-generation immunoassay methods (see online Supplemental Fig. 3).

**Discussion**

Our immunocapture in situ digestion LC-MS/MS assay is capable of quantifying 1–84 PTH across a concentration range of 39.1–4560 ng/L \((4–483 \text{ pmol/L})\) with high analytical specificity. The method combines immunocapture, protein cleavage with the protease trypsin, and LC-MS/MS, all approaches that have been used recently to improve the quantification of peptides and proteins within a clinical laboratory setting.

In fact, protein cleavage coupled with LC-MS/MS has been gaining favor for the absolute quantification of proteins ever since Barr et al. \((15)\) first described its use for quantifying apolipoprotein A-1 reference material more than 10 years ago. Since that time, other groups have shown that protein cleavage coupled with LC-MS/MS can be used to quantify proteins directly from serum without enrichment \((16–19)\).

In many cases, groups have also relied on immunocapture to enrich low-abundance proteins before
immunocapture approaches have been used before LC-MS/MS that function in a manner similar to the “bottom-up” and “top-down” approaches used in mass spectrometry–based proteomics workflows. The bottom-up methodology starts with a digested sample that is allowed to incubate with antibodies raised against specific proteolytic peptides before analysis by LC-MS/MS. This approach has been made popular by Anderson et al. (20), who were the first to successfully demonstrate its use for quantifying multiple proteins from serum. Recently, it was shown that immunofinity peptide enrichment–tandem mass spectrometry can detect tryptic peptides of thyroglobulin at picomolar concentrations (21). The top-down approach starts by using antibodies to capture the intact protein before digestion. The immunocaptured protein can either be eluted off the antibody before digestion or the protein can be digested while attached to the antibody (i.e., in situ) (22).

We chose the top-down approach for PTH because the protein readily digests in situ without the need for additional steps such as reduction and alkylation. Also, the likelihood of interference by PTH fragments with an intact N-terminus is reduced, because the monoclonal antibody on the Immulite bead was generated using the C-terminal portion (amino acids 44–84) of PTH. Furthermore, we took full advantage of stable isotope–labeled recombinant PTH (15NrpPTH) as an internal standard, which allowed for correction of losses occurring during various steps of the method.

Substantial variability and bias is commonly observed in the commercial automated sandwich immunoassays. Thorough understanding of the interferences and cross-reactivities with PTH fragments in these commercial assays is lacking (8–11). Differences in PTH results were also observed between the LC-MS/MS and the Scantibodies third-generation immunoassay method (see online Supplemental Fig. 3). A detailed investigation will be required to identify various PTH fragments that could be sources for bias among various PTH assays.

In the LC-MS/MS method, all the tested PTH fragments and PTHrP showed cross-reactivities between 0.1% and ≤0.001%, consistent with instrument background signals. Most notably, the 7–84 PTH fragment, which is captured by the antibody, shows no cross-reactivity owing to the specific nature of the MS/MS detection of the tryptic 1–13 PTH fragment (1SV SEIQLHMNLGK13). This tryptic PTH fragment shares only 6 and 7 amino acids with N-terminal tryptic peptides of PTHrP and 7–84 PTH, respectively, and has no substantial homology with any other trypsin digestion fragments of human proteins, making it a highly specific marker for 1–84 PTH. The key feature of the current LC-MS/MS assay is that it can detect longer N-terminal PTH fragments (>1–44) in addition to 1–84 PTH.

Recent data from the US Renal Data System on patients with chronic kidney disease and end-stage renal disease (ESRD) have shown that increasing rates of parathyroidectomies and potentially inappropriate treatment with calcitriol or its analogs may be associated with the use of PTH immunoassays that show cross-reactivity with 7–84 PTH and other interfering PTH fragments. In fact, a recent comparison of PTH values obtained on various commercial PTH assays showed that some immunoassays fail to categorize pa-
tients with ESRD into appropriate calcitriol treatment groups (23–25). Finally, there remains a dire need for a reliable reference methodology to verify calibrators and assay performance, particularly for patients with ESRD.

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**References**