

# Selected-Reaction Monitoring–Mass Spectrometric Immunoassay Analysis of Parathyroid Hormone and Related Variants

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## Key Words

LTQ Orbitrap XL, TSQ Vantage, Pinpoint Software, parathyroid hormone, PTH, biomarkers, MSIA

## Goal

To develop a highly sensitive and selective selected-reaction monitoring–mass spectrometric immunoassay analysis (SRM-MSIA)-based method for the concurrent detection and quantification of full-length parathyroid hormone (PTH) [amino acid (aa)1–84] and two N-terminal variants [aa7–84 and aa34–84] for clinical research use.

## Introduction

Parathyroid hormone is produced in the parathyroid glands through the two-step conversion of prepro-PTH (115 amino acids) to pro-PTH (90 amino acids) to the 84 amino acid peptide (PTH1–84). Conventional PTH measurements typically rely on two-antibody recognition systems coupled to a variety of detection modalities.<sup>1</sup> The most specific modalities are able to differentiate between different truncated forms of PTH and are referred to as second- and third-generation PTH assays.<sup>2</sup> The key to the application of these later-generation assays is the ability to selectively detect and quantify various PTH forms. In particular, two variants are the subject of increased research investigation: full-length PTH1–84 and PTH missing the 6 N-terminal amino acids (PTH7–84). Because of the inability of existing tests to detect microheterogeneity,<sup>3</sup> these variants were historically considered as a single PTH value (by the first-generation assays). The classification of each variant as its own molecular entity, and the analysis of each independently, suggest an antagonistic relationship between the two different forms in regard to calcium homeostasis.<sup>4</sup> In fact, there is mounting research showing that the ratio between PTH1–84 and PTH7–84 could have future clinical relevance for distinguishing between hyper-parathyroid bone turnover and adynamic bone disease.<sup>5–7</sup>



The ratio of PTH1–84 to PTH7–84 is an example of the potential utility of the microheterogeneity within the PTH protein. Another PTH variant, PTH1–34, has been identified as exhibiting biochemical activity comparable to the full-length protein. There are indications that the microheterogeneity of PTH has yet to be fully characterized, challenging researchers' efforts to determine the utility and/or confounding effects on present-day methods. Accurate examination of known PTH variants and the simultaneous evaluation of other possible variants requires a degree of analytical freedom that universally escapes conventional methods. This work describes mass spectrometric immunoassays that, although specifically designed for the detection of PTH1–84 and PTH7–84, also facilitate the simultaneous discovery and evaluation of further microheterogeneity in PTH.

## Experimental

### Approach

In addition to the well-characterized truncated PTH variants, PTH1–84 and PTH7–84, four other molecular versions have been reported in the literature as present in human biofluids (primarily plasma or serum). Aligning these fragments to the sequence of PTH1–84 produced a variant map revealing forms stemming predominantly from N-terminal truncations (Figure 1). A conserved region (among several variants) was evident between residues 48 and 84. This region was suitable for immunoaffinity targeting to capture ragged N-terminal variants (for example, PTH1–84 and PTH7–84). Postcapture digestion of retained PTH (and variants) created the basis for SRM-MSIA,<sup>8–11</sup> for which surrogate peptides representative of the different PTH variants were selected for analysis.

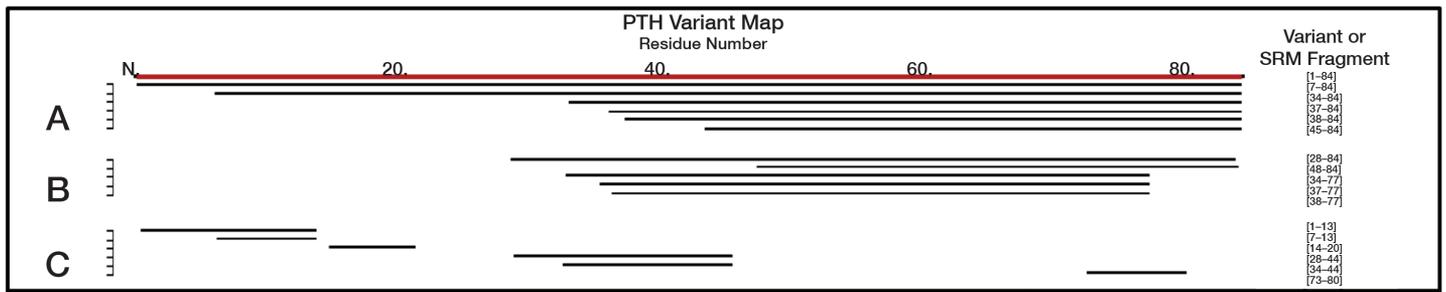


Figure 1. PTH variant map. (A) N-terminally truncated PTH variants identified previously.<sup>7,12</sup> (B) Variants added to map by top-down MS analysis. (C) Conserved and truncated tryptic fragments chosen for SRM-MSIA.

### Reagents

Goat polyclonal anti-PTH39–84 antibody was purchased from Immutopics International. Recombinant human PTH (rhPTH) was obtained from Bachem. Premade 0.01 M HEPES-buffered saline with 3 mM EDTA and 0.05% (vol/vol) surfactant P20 (HBS-EP) was purchased from Biacore. Thermo Scientific™ Pierce™ premixed 2-[morpholino]ethanesulfonic acid–buffered saline powder packets and Thermo Scientific synthetic heavy-labeled peptides were used. High purity solvents from Fisher Chemical brand were used.

### Samples

A total of 24 plasma samples were used in the research study: 12 from individuals with previously diagnosed severe renal impairment or end-stage renal disease (ten males and two females; mean age 66.7 years) and 12 from healthy individuals (ten males and two females; mean age 65 years). Among the individuals with renal failure, three were Hispanic, two were Asian, two were African American, and six were Caucasian. The ethnicity information for the healthy sample donors was not available.

### Calibration Curves Samples

Samples for creation of calibration curves were prepared from pooled human plasma by step-wise, 2-fold serial dilution of an initial sample containing rhPTH at a concentration of 1000 ng/L (eight steps, range 1000–7.8 ng/L). Samples were frozen at -80 °C until use.

### Sample Preparation and Immunocapture

Purification and concentration of the PTH was accomplished by immunoaffinity capture. Extraction of PTH from plasma was carried out with proprietary Thermo Scientific™ Mass Spectrometric Immunoassay (MSIA™) pipette tips derivatized with the PTH antibodies via 1,1 -carbonyldiimidazole chemistry.<sup>13–17</sup> After extraction, PTH was digested, separated by liquid chromatography, and analyzed by high-resolution MS/MS on an ion trap-Orbitrap™ hybrid mass spectrometer and by SRM on a triple quadrupole mass spectrometer as described below.

### Sample Elution and Trypsin Digestion

Bound proteins were eluted from the tips into a 96 well plate by pipetting 100 µL of 30% acetonitrile/0.5% formic acid up and down for a total of 15 cycles. Samples were lyophilized to dryness and then resuspended in 30 µL of 30% n-propanol/100 mmol/L ammonium bicarbonate, pH 8.0, diluted with 100 µL of 25 M acetic acid containing 100 ng of trypsin. Samples were allowed to digest for 4 hours at 37 °C. After digestion, samples were lyophilized and resuspended in 30 µL of 3% (vol/vol) acetonitrile/0.2% (vol/vol) formic acid/glucagon/PTH heavy peptides.

## High-Resolution LC-MS/MS

High-resolution LC-MS/MS analysis was carried out using a Thermo Scientific™ EASY-nLC™ system and Thermo Scientific™ LTQ Orbitrap XL™ hybrid ion trap-Orbitrap mass spectrometer. Samples in 5% (vol/vol) acetonitrile/0.1% (vol/vol) formic acid were injected into a Thermo Scientific™ Hypersil GOLD™ aQ fused-silica capillary column (75 μm x 25 cm, 5 μm particle size) in a 250 μL/min gradient of 5% acetonitrile/0.1% formic acid to 30% acetonitrile/0.1% formic acid over the course of 180 minutes. The total run time was 240 minutes and the flow rate was 285 nL/min. The LTQ Orbitrap XL MS was operated at 60,000 resolution (FWHM at  $m/z$  400) for a full scan for data-dependent Top 5 MS/MS experiments (CID or HCD). The top 5 signals were selected with monoisotopic precursor selection enabled, and +1 and unassigned charge states rejected. Analyses were carried out in the ion trap or the Orbitrap analyzer. The experiments were performed using collision-induced dissociation (CID) and higher-energy collisional dissociation (HCD) fragmentation modes.

## SRM Methods

SRM methods were developed on a Thermo Scientific™ TSQ Vantage™ triple stage quadrupole mass spectrometer with a Thermo Scientific™ Accela™ pump, a CTC PAL® autosampler (Leap Technologies), and a Thermo Scientific™ Ion Max™ source equipped with a high-flow metal needle. A mass window of 0.7 full width at half maximum (FWHM, unit resolution) was used in the SRM assays because the immunoenriched samples had a very high signal-to-noise ratios. Narrower windows were necessary when the matrix background was significant and caused interferences that reduced signal-to-noise in the SRM channels. Reversed-phase separations were carried out on a Hypersil GOLD column (1 mm x 100 mm, 1.9 μm particle size) with a flow rate of 160 μL/min. Solvent A was 0.2% formic acid in LC-MS-grade water, and solvent B was 0.2% formic acid in Fisher Scientific™ Optima™-grade acetonitrile.

## Software

Thermo Scientific™ Pinpoint™ software was used for targeted protein quantification, automating the prediction of candidate peptides and the choice of multiple fragment ions for SRM assay design. Pinpoint software was also used for peptide identity confirmation and quantitative data processing. The intact PTH sequence was imported into the software and digested with trypsin *in silico*. Then, transitions for each peptide were predicted and tested with recombinant PTH digest to determine those peptides and transitions delivering optimal signal. After several iterations, a subset of six peptides with multiple transitions was chosen.

Further tests were conducted with this optimized method. After the target peptides were identified, heavy arginine or lysine versions were synthesized to be used as internal quantitative standards. Target peptides were subsequently identified and quantified by coeluting light- and heavy-labeled transitions in the chromatographic separation. Time alignment and relative quantification of the transitions were performed with Pinpoint software. All samples were assayed in triplicate.

## Results and Discussion

### Top-Down Analysis and Discovery of Novel Variants

The approach described herein coupled targeting a common region of PTH by use of a polyclonal antibody (raised to the C-terminal end of the protein) with subsequent detection by use of SRM MS. Numerous PTH variants were simultaneously extracted with a single, high-affinity polyclonal antibody, and the selection of the epitope was directed by the target of interest (i.e., intact and N-terminal variants). The primary goal was to differentiate between intact PTH1–84 and N-terminal variant PTH7–84 while simultaneously identifying any additional N-terminal heterogeneity throughout the molecule. The results of these top-down experiments allowed the development of an initial standard profile for PTH. Clearly, this profile is not finite, and may be expanded to include additional variants found through literature search and/or complementary full-length studies. However, this standard profile provided an initial determination of target sequences for developing specific SRM assays.

### Selection of Transitions for SRM

During LC-MS/MS analysis, multiple charge states and fragmentation ions were generated from each fragment, resulting in upwards of 1000 different precursor/product transitions possible for PTH digested with trypsin. Empirical investigation of each transition was not efficient. Therefore, a workflow incorporating predictive algorithms with iterative optimization was used to predict the optimal transitions for routine monitoring of tryptic fragments (Figure 2). The strategy facilitated the translation of peptide intensity and fragmentation behavior empirically obtained by high-resolution LC-MS/MS analyses to triple quadrupole SRM assays. Inherent to the success of the workflow was the similarity of peptide ion fragmentation behavior in these ion trap and triple quadrupole instruments.<sup>12</sup> Empirical data from such LC-MS/MS experiments were used in conjunction with computational methods (*in silico* tryptic digestions and prediction of SRM transitions) to enhance the design of effective SRM methods for selected PTH peptides.

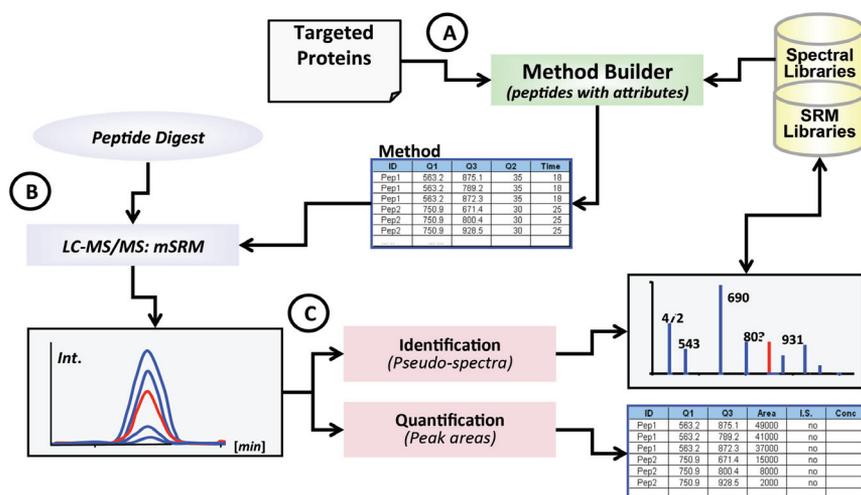


Figure 2. Pinpoint workflow for development of multiplexed SRM assays.

[Q = quadrupole; mSRM = multiple SRM; Int. = intensity; I.S. = internal standard; Conc = concentration. Time measurements are in minutes (min).]

The initial list of transitions was queried empirically to produce an LC-MS/MS profile based on four tryptic peptides that collectively spanned >50% (45 of 84 amino acids) of the full PTH sequence. SVSEIQLMHNLGK [amino acid (aa)1–13] was monitored to represent PTH species with an intact N-terminus, such as PTH1–84. Other tryptic peptides, HLNSMER (aa14–20), DQVHNFVALGAPLAPR (aa28–44), and ADVNVLTK (aa73–80) were included for monitoring across the PTH sequence. In addition, transitions for two truncated tryptic peptides, LMHNLGK (aa7–13) and FVALGAPLAPR (aa34–44), were added to the profile to monitor for truncated variants PTH7–84 and PTH34–84, respectively. In total, 32 SRM transitions tuned to these six peptides were used to monitor intact and variant forms of PTH (Figure 1).

### Generation of Standard Curves and Limits of Detection and Quantification

rhPTH was spiked into stock human blood plasma to create calibration curves for all target tryptic peptides through serial dilution. As illustrated in Figure 3 for peptides LQDVHNFVALGAPLAPR (aa28–44) and SVSEIQLMHNLGK (aa1–13), SRM transitions for the four wild-type tryptic fragments exhibited linear responses ( $R^2 = 0.90–0.99$ ) relative to rhPTH concentration, with limits of detection for intact PTH of 8 ng/L and limits of quantification for these peptides calculated at 31 and 16 ng/L, respectively. Standard error of analysis for all triplicate measurements in the curves ranged from 3% to 12% for all peptides, with <5% chromatographic drift between replicates. In addition, all experimental peptide measurements were calculated relative to heavy-labeled internal standards. CVs of integrated areas under the curve for 54 separate measurements (for each heavy peptide) ranged from 5% to 9%. Monitoring of variant SRM transitions showed no inflections relative to rhPTH concentration, owing to the absence of truncated variants in the stock rhPTH.

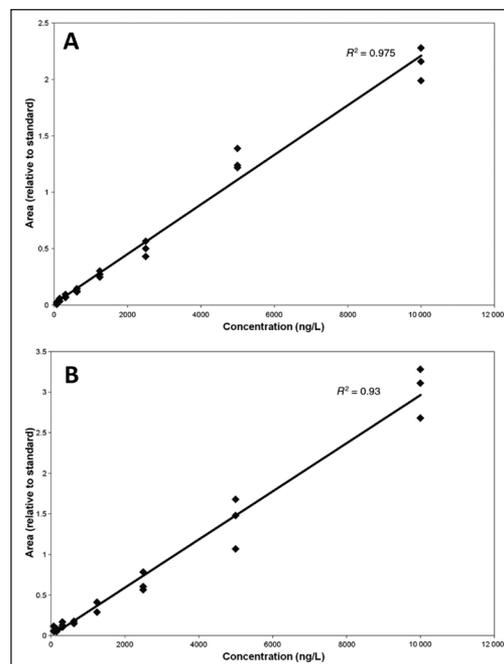


Figure 3. SRM calibration curves for PTH peptides. (A) Peptide LQDVHNFVALGAPLAPR aa28–44. (B) Peptide SVSEIQLMHNLGK aa1–13.

### Evaluation of Research Study Samples

Initial SRM data were acquired from replicate plasma samples. The light and heavy peptides coeluted precisely in all samples. Further SRM experiments were carried out on the cohort of renal failure ( $n = 12$ ) and normal ( $n = 12$ ) samples. The most prominent PTH variant in the renal failure samples was PTH34–84. To quantify this observation with SRM, all samples were interrogated to determine the expression ratios of renal failure to normal for the various target peptides, including FVALGAPLAPR (aa34–84), which should be specific to the 34–84 variant. Chromatographic data from single renal-failure samples for peptides FVALGAPLAPR (aa34–44) and SVSEIQLMHNLGK (aa1–13) are shown in Figure 4. The peak integration area and individual coeluting fragment transitions for each peptide are illustrated. Similar chromatograms were obtained for peptides LQDVHNFVALGAPLAPR (aa28–44), HLNSMER (aa14–20), and ADVNVLTK (aa73–80) (data not shown). The sample variances and expression ratios of renal-failure samples to normal samples for each peptide are shown in Figure 5. The expression ratios for the peptides ranged from 4.4 for FVALGAPLAPR (aa34–44) to 12.3 for SVSEIQLMHNLGK (aa1–13). Notable quantities of peptide LMHNLGK (aa 7–13) were not detected in these samples. Sample variances illustrated in the scatter plots in Figure 5 demonstrate that the renal failure and normal samples groups were clearly segregated by the five target peptides.

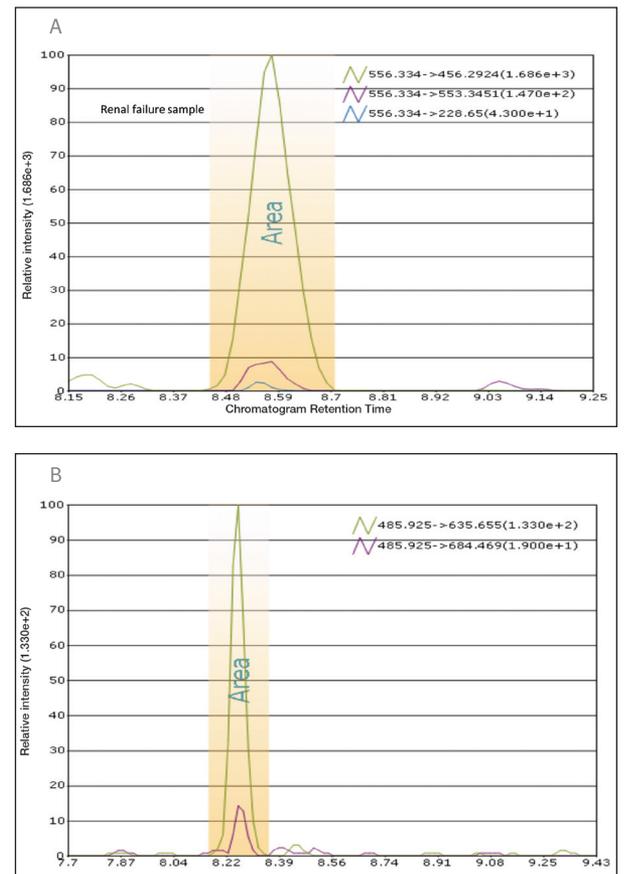


Figure 4. Pinpoint software SRM data from samples of normal and renal failure patients. Chromatographic data illustrate peak integration area and individual fragment transitions for peptides from single renal failure samples. (A) Semitryptic peptide FVALGAPLAPR (aa34–44), specific to the 34–84 variant (see Figure 1). (B) Tryptic peptide SVSEIQLMHNLGK (aa1–13).

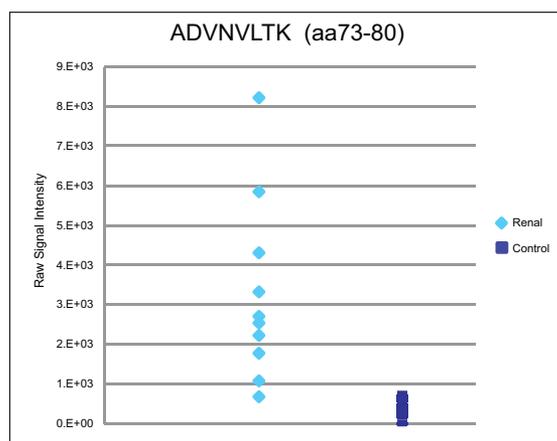
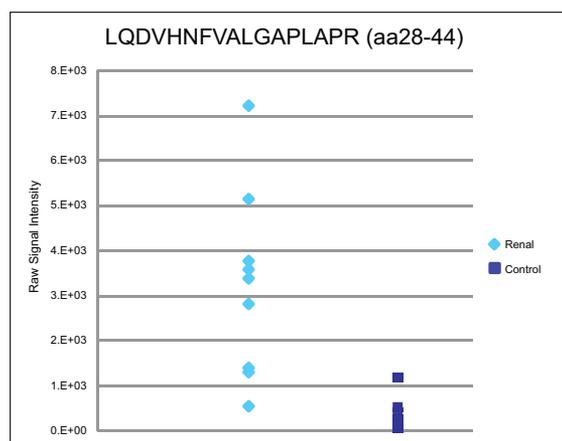


Figure 5. SRM quantitative ratios and sample variances of PTH peptides in samples from renal failure patients (Renal) and healthy controls. Ratios refer to the average value of the renal cohort divided by the average value of the healthy control cohort.

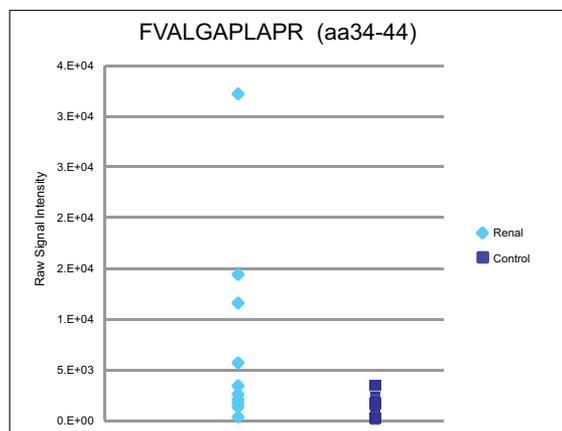
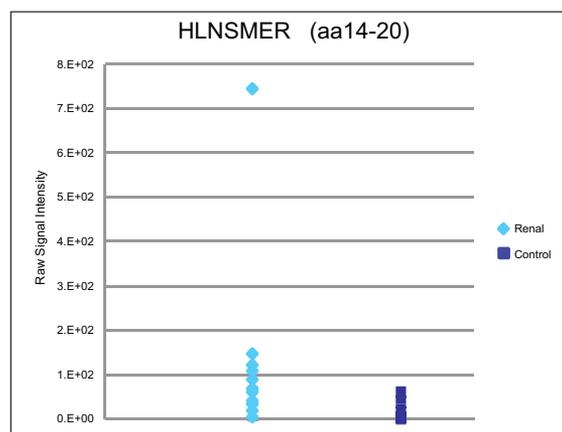
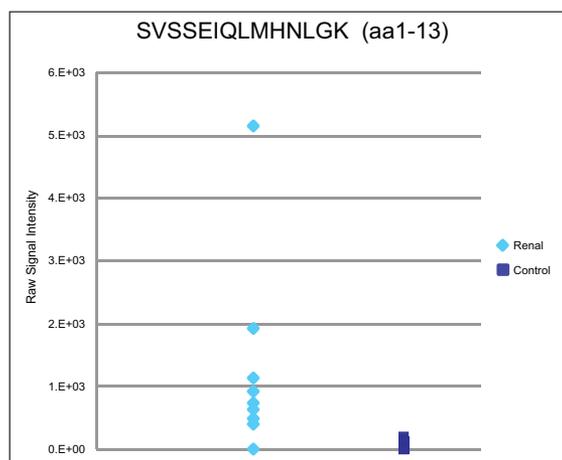
### Conclusion

An SRM-MSIA-based analysis method was developed capable of simultaneously monitoring full-length PTH and truncated variants with analytical metrics suitable for clinical research use. Using a workflow incorporating postcapture tryptic digestion, surrogate peptides representative of PTH1–84 and PTH7–84 were generated and then monitored using SRM. In addition, tryptic fragments spanning other regions of PTH were incorporated into the analysis. Relative ion signals for these species confirmed that the clinical research method was functional and created the basis for a standard PTH profile. This standard profile was expanded to include a peptide representative of a novel variant, PTH34–84, clipped at the N-terminus. In total, 32 SRM transitions were analyzed in a multiplexed method to monitor nonvariant PTH sequences with >50% sequence coverage, as well as the two truncated variants. Peptides exhibited linear responses ( $R^2 = 0.90\text{--}0.99$ ) relative to the limit of detection for an intact recombinant human PTH concentration of 8 ng/L. Limits of quantification were 16–31 ng/L, depending on the peptide. Standard error of analysis for all triplicate measurements was 3%–12% for all peptides, with <5% chromatographic drift between replicates. The CVs of integrated areas under the curve for 54 separate measurements of heavy peptides were 5%–9%.

Pinpoint software was used to develop and implement “intelligent SRM” data acquisition strategies, increasing instrument efficiency by avoiding the need to monitor all of the specified transitions at all times. Use of these techniques may be particularly advantageous for clinical research laboratories in methods where a large number of PTH variants are monitored, or where the analyzed sample contains a complex mixture of PTH-derived peptides and components produced by digestion of compounds in the sample matrix.

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