Stable-Isotope-Labeled Parathyroid Hormone as an Immunocapture and Digestion-Efficiency Internal Standard for MS-Based Immunoassays

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Overview

Purpose: Apply a mass spectrometric immunoassay-selective reaction monitoring (MSIA-SRM) assay to measure intact and truncated parathyroid hormone (PTH) isoforms in plasma using a stable-isotope-labeled (heavy) protein standard for control of immunocapture and digestion efficiency.

Methods: Recombinant heavy PTH was expressed using a novel human cell-free, in vitro translation (IVT) system. Sample preparation and MSIA-SRM targeted quantitation were as previously described.1

Results: Heavy PTH with >95% isotope incorporation was expressed using a human IVT system and used to assess MSIA capture efficiency.

Introduction

Parathyroid hormone (PTH) assays for monitoring PTH and PTH variants are important for the accurate diagnosis of endocrine and osteological diseases. The heterogeneity of PTH has traditionally been an impediment to the development of assays that distinguish full-length PTH (PTH1-84) from N-terminally truncated PTH (PTH 7-84 and others) (Figure 1A). Because intact and truncated forms of PTH vary in biological activity, assays that can accurately quantify the ratio of intact hormone to its fragments are needed to accurately determine the amount of biologically active PTH. To date, most immunoassays used to monitor PTH levels are based on traditional sandwich ELISA methods that cannot accurately discriminate intact PTH from truncated PTH. In addition, these methods typically employ primary antibodies to the N-terminus of the hormone, thereby preventing quantification of any fragments. To monitor the numerous isoforms of PTH, we used a mass spectrometric immunoassay (MSIA) for targeted enrichment and quantitation of PTH and PTH variants using selected reaction monitoring (SRM) (Figure 2).1 In this study, we also expressed and characterized a stable-isotope-labeled PTH protein for use as an internal standard for immunocapture and digestion efficiency.

Methods

Heavy PTH expression and purification: Full-length PTH (1-115 AA) was expressed as a C-terminal 6xHIS fusion using the Thermo Scientific 1-Step Heavy Protein IVT Kit. Expressed protein was purified using a Thermo Scientific HisPur Cobalt Purification Kit under denaturing conditions (8M urea in Binding buffer) and refolded on the column before imidazole elution.

Heavy PTH MS analysis: For stable-isotope-incorporation determination, proteins were denatured, reduced, alkylated, digested with trypsin, and desalted. A Thermo Scientific LTQ Orbitrap XL mass spectrometer equipped with electron transfer dissociation (ETD) was used for peptide analysis and MS spectra were searched against a custom PTH FASTA database using Thermo Scientific Proteome Discoverer software version 1.3 with SEQUEST®. Static modifications included carbamidomethyl, lysine-8, and arginine-10. Methionine oxidation was used as the dynamic modification.

Results

Previously, we developed multiplexed SRM assays for PTH that allow for quantification of four fully tryptic peptides (spanning the entire PTH sequence) and two semi-tryptic, variant-specific peptides.1 2 With this approach, we could monitor intact PTH and N-terminally truncated PTH isoforms with high sensitivity.

To quantitatively assess sample preparation steps for MS immunoassays, we produced and characterized a stable-isotope-labeled PTH protein as an internal standard. A DNA construct of PTH encoding the hormone sequence with a novel quantitative peptide sequence and a C-terminal 6xHis-tag was used for recombinant protein expression with a human IVT kit (Figure 3). Light and heavy versions of PTH were expressed at high levels (>50 µg/mL) and highly purified using a cobalt immobilized metal affinity column (IMAC) (Figures 3C and 3D).

Heavy-PTH isotope incorporation efficiency was assessed using high-resolution mass spectrometry and measured to be 96.2% heavy-protein labeled based on the average of identified PTH peptides (Figure 3E). Light and heavy PTH was combined to demonstrate excellent linearity of quantitation (Figure 4). Heavy PTH was successfully captured using anti-PTH MSIA pipette columns and analyzed using a previously described targeted SRM assay (Figure 5). Research continues on immunocapture and digestion efficiency of heavy and light PTH in neat and serum samples.
Stable-Isotope-Labeled Parathyroid Hormone as an Immunocapture and Digestion-Efficiency Internal Standard for use as an internal standard for immunocapture and digestion efficiency.

Heavy and light PTH in neat and serum samples. Excellent linearity of quantitation (Figure 4). Heavy PTH was successfully captured using identified PTH peptides (Figure 3E). Light and heavy PTH was combined to demonstrate spectrometry and measured to be 96.2% heavy-protein labeled based on the average of Heavy-PTH isotope incorporation efficiency was assessed using high-resolution mass (IMAC) (Figures 3C and 3D).

Sequence and a C-terminal 6xHis-tag was used for recombinant protein expression with construct of PTH encoding the hormone sequence with a novel quantitative peptide terminally truncated PTH isoforms with high sensitivity.

Methionine oxidation was used as the dynamic modification. Variant-specific peptides.1,2 With this approach, we could monitor intact PTH and N-terminally truncated PTH isoforms with high sensitivity.

Heavy PTH MS analysis:

Before imidazole elution.

Under denaturing conditions (8M urea in Binding buffer) and refolded on the column

Heavy PTH expression and purification:

A C-terminal 6xHIS fusion using the Thermo Scientific 1-Step Heavy Protein IVT Kit.

Heavy PTH with >95% isotope incorporation was expressed using a human IVT system and used to assess MSIA capture efficiency.

Methods:

Apply a mass spectrometric immunoassay-selective reaction monitoring (MSIA-SRM) assay to measure intact and truncated parathyroid hormone (PTH) isoforms.

Purpose:

Overview

Analyses of PTH in clinical samples revealed a large degree of heterogeneity and truncated variants, principally at the amino terminus.1-2 B) In order to quantify intact and truncated forms, we chose four fully tryptic and two semi-tryptic (variant-specific) peptides for the multiplexed SRM assay.

FIGURE 1. PTH protein variant map. PTH is secreted by the parathyroid gland as an 84-amino-acid polypeptide. The PTH protein active site is from aa1-10. N-terminally truncated variants may confound immunoassays that do not distinguish intact from truncated forms of the protein. A) Previous top-down analyses of PTH in clinical samples revealed a large degree of heterogeneity and truncated variants, principally at the amino terminus.1-2 B) In order to quantify intact and truncated forms, we chose four fully tryptic and two semi-tryptic (variant-specific) peptides for the multiplexed SRM assay.

FIGURE 2. MSIA-SRM workflow for enrichment and quantification of low-abundance proteins. The wide dynamic range of proteins in blood presents a technical hurdle for the development of low-abundance analytes. The reference range for PTH is 1 pmol/L or 10-60 pg/mL (10-60 ng/L), making it one of the least abundant clinically important analytes. Anti-PTH antibodies immobilized on pipette-tip microcolumns were used for PTH immunocapture and enrichment in conjunction with an automated liquid handling (ALH) Versette platform for high-throughput sample processing. Immunocaptured-PTH was quantified by SRM mass spectrometry using a Thermo Scientific TSQ Vantage triple quadrupole mass spectrometer. Using this workflow for protein-based immuno-enrichment, both full-length PTH and PTH isofom variants can be monitored using different targeted peptides (Figure 1).
Methionine oxidation was used as the dynamic modification. Heavy and light PTH in neat and serum samples. Research continues on immunocapture and digestion efficiency of anti-PTH MSIA pipette columns and analyzed using a previously described targeted SRM assay (Figure 5). Heavy PTH expression and purification:

A DNA translation (IVT) system. Sample preparation and MSIA-SRM targeted quantitation system and used to assess MSIA capture efficiency. In order to characterize a stable-isotope-labeled PTH protein as an internal standard. A DNA sequence and a C-terminal 6xHis-tag was used for recombinant protein expression with and purified using a cobalt immobilized metal affinity column under denaturing conditions (8M urea in Binding buffer) and refolded on the column then purified and digested into peptides for LC-MS analysis. Percent incorporation of stable-isotope-labeled PTH peptides SVSEIQLMHNLGK and SLGEADKADVNLTK. Quantitation for each peptide is shown in the graphs below the spectra.

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FIGURE 2. MSIA-SRM workflow for enrichment and quantification of low-abundance analytes. The reference translation (IVT) system. Sample preparation and MSIA-SRM targeted quantitation system and used to assess MSIA capture efficiency. In order to characterize a stable-isotope-labeled PTH protein as an internal standard. A DNA sequence and a C-terminal 6xHis-tag was used for recombinant protein expression with and purified using a cobalt immobilized metal affinity column under denaturing conditions (8M urea in Binding buffer) and refolded on the column then purified and digested into peptides for LC-MS analysis. Percent incorporation of stable-isotope-labeled PTH peptides SVSEIQLMHNLGK and SLGEADKADVNLTK. Quantitation for each peptide is shown in the graphs below the spectra.

FIGURE 3. Recombinant PTH expression, purification and MS analysis A) IVT lysates are combined with the reaction mixture, vector DNA and stable-isotope-labeled amino acids to express recombinant proteins. B) Expressed proteins are then purified and digested into peptides for LC-MS analysis. Percent incorporation of stable-isotopes is based on ratio of areas under the curve (AUC) for heavy and light stable-isotope-labeled peptides. C) Relative expression of recombinant light and heavy PTH compared to a GFP control as shown by anti-6xHis Western blot analysis. D) Coomassie-stained SDS-PAGE gel of cobalt purified PTH labeled with heavy lysine and heavy arginine. E) Representative MS spectra of stable-isotope-labeled PTH peptides SVSEIQLMHNLGK and SLGEADKADVNLTK. Quantitation for each peptide is shown in the graphs below the spectra.

B) In order to characterize a stable-isotope-labeled PTH protein as an internal standard. A DNA sequence and a C-terminal 6xHis-tag was used for recombinant protein expression with and purified using a cobalt immobilized metal affinity column under denaturing conditions (8M urea in Binding buffer) and refolded on the column then purified and digested into peptides for LC-MS analysis. Percent incorporation of stable-isotope-labeled PTH peptides SVSEIQLMHNLGK and SLGEADKADVNLTK. Quantitation for each peptide is shown in the graphs below the spectra.

C) Relative expression of recombinant light and heavy PTH compared to a GFP control as shown by anti-6xHis Western blot analysis. D) Coomassie-stained SDS-PAGE gel of cobalt purified PTH labeled with heavy lysine and heavy arginine. E) Representative MS spectra of stable-isotope-labeled PTH peptides SVSEIQLMHNLGK and SLGEADKADVNLTK. Quantitation for each peptide is shown in the graphs below the spectra.
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Methionine oxidation was used as the dynamic modification. Heavy and light PTH in neat and serum samples.

Anti-PTH MSIA pipette columns and analyzed using a previously described targeted SRM identified PTH peptides (Figure 3E). Light and heavy PTH was combined to demonstrate sequence and a C-terminal 6xHis-tag was used for recombinant protein expression with translation (IVT) system. Sample preparation and MSIA-SRM targeted quantitation of PTH and PTH variants using selected reaction monitoring (SRM) (Figure 1B).

Results

Overview

Heavy PTH expression and purification:

Full-length PTH (1-115 AA) was expressed as

immuno-enrichment, both full-length PTH and PTH isoform variants can be

triple quadrupole mass spectrometer. Using this workflow for protein-based

analyses of PTH in clinical samples revealed a large degree of heterogeneity

N-terminally truncated variants may confound immunoassays that do not

quantify intact and truncated forms, we chose four fully tryptic and two semi-

tryptic, variant-specific peptides.1,2 With this approach, we could monitor intact PTH and N-

four fully tryptic peptides (spanning the entire PTH sequence) and two semi-

immunoassays used to monitor PTH levels are based on traditional sandwich ELISA accurately determine the amount of biologically active PTH. To date, most

PTH has traditionally been an impediment to the development of assays that distinguish

immunoassays.

Apply a mass spectrometric immunoassay-selective reaction monitoring

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FIGURE 4. Ten point calibration curve ranging from 0-2000pg/mL for the N-terminal PTH peptide SVSEIOLMHNLGK. CV’s of triplicate points were 1-19%, and the R² was 0.978. Calibration curves for the peptides HLNSMER, ADVNLTK and LQDVHNFVALGAPLAPR demonstrated similar linearity and precision.

FIGURE 5. SRM quantitation of light and heavy PTH peptides. Endogenous or heavy PTH spiked into plasma was immunoprecipitated using the MSIA anti-PTH pipette tips. Light and heavy PTH peptide signals were monitored by SRM using the peptide transitions described in Figure 1B.
Conclusion

- The MSIA-SRM assay can be used to measure physiological PTH concentrations.
- Heavy PTH with >95% isotope incorporation was expressed using a human IVT system.
- Heavy PTH was used to assess MSIA capture efficiency and can be used to measure digestion efficiency.

References

