New CDC standardization projects on PTH and thyroid function tests

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Overview

- Current Challenges in PTH Testing
- Developments to the CDC PTH Reference Method
- CDC’s New Thyroid Reference Methods
- Future Directions
Studies suggest that current inaccuracy among PTH assays can lead to inconsistent diagnosis

3.4 fold difference

Blood contains intact PTH and a range of different PTH fragments/modifications that can affect measurement accuracy

Some ratio of PTH fragments to PTH(1-84) may have potential for use in the diagnosis of:
- Hyperparathyroid-Associated Bone Loss
- Non-Dynamic Bone Disease
- Severe or End-stage Renal Disease

**PTH Fragment Type** | **Amino Acid Range**
--- | ---
**Intact** | 1-84
**N-terminal** | 1-34 1-44
**Mid-range** | 7-34 34-77 35-64 37-77 38-77 44-68
**C-terminal** | 4-84 7-84 28-84 34-84 35-84 37-84 38-84 45-84 48-84 53-84

*Phosphorylation and oxidation can occur at serine and methionine*
The candidate reference measurement procedure needs to meet specific criteria that are specific to PTH testing

### Method Criteria

- Specific for intact PTH without interference
- Able to detect PTH fragments
- Measure PTH in native blood samples
- Meet ISO and other relevant standards

### Laboratory Approach

- UHPLC/MS/MS with stable-isotope dilution quantitation
- Isolation of the analyte from sample matrix
- Use certified reference materials for traceability

### Desirable Specification

<table>
<thead>
<tr>
<th>Specimen</th>
<th>Imprecision</th>
<th>Inaccuracy</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum</td>
<td>6-7%</td>
<td>4-5%</td>
</tr>
<tr>
<td>Plasma</td>
<td>6-7%</td>
<td>6-7%</td>
</tr>
</tbody>
</table>

Requirements are based on biological variability from the references listed.

Low endogenous concentrations of PTH in serum require highly sensitive and specific methodologies.

High-Resolution Mass Spectrometry allows for excellent intact PTH specificity.

Full MS Scan
- Single Donor Serum (1 mL)
- Sample Pre-treatment

SIM Window
- m/z 1569.835-1573.835

EIC for PTH in SIM Window
- m/z 1571.83525
- 5 ppm mass accuracy
An immunocapture strategy is being evaluated as part of the sample preparation workflow for the PTH Reference Method.

Sample Collection
- Single-donor serum

1 mL

Internal Standard Addition
- Spike in the following:
  1. [¹⁵N] Stable-Isotope Labeled Internal Standards
  2. PBS/BSA Buffer

750 μL

PTH Isolation from Matrix

80 μL

PTH Elution
- Perform Elution of PTH from MSIA-Tips into 96 well plates

80 μL

Mass Spectral Analysis of Intact PTH
- UHPLC-HRMS analysis of Intact PTH without enzymatic digestion

Adapted from Lopez, M. F., et al. Clinical Chemistry 2010, 56, 281-290
Initial assessments demonstrate excellent linearity, reproducibility, and sufficient sensitivity of the UHPLC-HRMS system.

Normal Biological Levels of PTH

<table>
<thead>
<tr>
<th></th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>iPTH</td>
<td>10 – 65 pg/mL</td>
</tr>
<tr>
<td>N-terminal</td>
<td>8 – 24 pg/mL</td>
</tr>
<tr>
<td>C-terminal</td>
<td>50 – 330 pg/mL</td>
</tr>
</tbody>
</table>

Target Levels of Intact PTH in KDOQI Guidelines for CKD-MBD

<table>
<thead>
<tr>
<th>CKD Stage</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stage 3</td>
<td>35 – 70 pg/mL</td>
</tr>
<tr>
<td>Stage 4</td>
<td>70 – 110 pg/mL</td>
</tr>
<tr>
<td>Stage 5</td>
<td>150 – 300 pg/mL</td>
</tr>
</tbody>
</table>

Preliminary Standard Curve for Intact PTH(1-84)

\[ y = 0.0003x + 0.0075 \]

\[ R^2 = 0.9967 \]

KDOQI Guidelines. NKF Am J Kidney Dis 2003, 42 (suppl 3), S1-201.
Immunoaffinity purification procedures allows for analysis of intact PTH and PTH-derived peptides in the same analytical run

<table>
<thead>
<tr>
<th>Fragment Name</th>
<th>Neutral m/z</th>
<th>RT (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>N-terminal</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PTH(1-34)</td>
<td>4115.1308</td>
<td>9.01</td>
</tr>
<tr>
<td>PTH(7-34)</td>
<td>3471.8131</td>
<td>8.77</td>
</tr>
<tr>
<td>PTH(28-44)</td>
<td>1816.9948</td>
<td>8.82</td>
</tr>
<tr>
<td>PTH(34-44)</td>
<td>1110.6549</td>
<td>8.66</td>
</tr>
<tr>
<td>PTH(34-77)</td>
<td>4712.4633</td>
<td>8.62</td>
</tr>
<tr>
<td>PTH(37-77)</td>
<td>4395.2894</td>
<td>8.37</td>
</tr>
<tr>
<td>PTH(38-77)</td>
<td>4282.2053</td>
<td>8.12</td>
</tr>
<tr>
<td>Mid-Range</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PTH(7-84)</td>
<td>8775.6468</td>
<td>8.87</td>
</tr>
<tr>
<td>PTH(28-84)</td>
<td>6175.2525</td>
<td>8.67</td>
</tr>
<tr>
<td>PTH(34-84)</td>
<td>5468.9127</td>
<td>8.60</td>
</tr>
<tr>
<td>PTH(35-84)</td>
<td>5321.8443</td>
<td>8.50</td>
</tr>
<tr>
<td>PTH(37-84)</td>
<td>5151.7387</td>
<td>8.45</td>
</tr>
<tr>
<td>PTH(38-84)</td>
<td>5038.6547</td>
<td>9.66</td>
</tr>
<tr>
<td>PTH(45-84)</td>
<td>4376.2683</td>
<td>9.35</td>
</tr>
<tr>
<td>PTH(48-84)</td>
<td>4133.1828</td>
<td>8.92</td>
</tr>
<tr>
<td>C-terminal</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PTH(7-84)</td>
<td></td>
<td></td>
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<tr>
<td>PTH(28-84)</td>
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<tr>
<td>PTH(48-84)</td>
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</tbody>
</table>

Non-labeled Intact PTH

[15N]-Labeled Intact PTH

Intact PTH m/z 1571.8352 z = 6

Intact [15N]-PTH m/z 1592.6045 z = 6
Inaccurate free T4 measurements may lead to incorrect diagnosis of patients

Mean deviations from the reference value for 4 serum concentration groups measured with 13 immunoassays

CDC is establishing a new reference methods for free and total T4 using equilibrium dialysis combined with UHPLC-MS/MS

Equilibrium Dialysis
- 5 kDa regenerated cellulose membranes
- PTFE 1 mL cells
- HEPES dialysis buffer (pH 7.5 ± 0.03 at RT)
- Dialysis at 37°C/4 hr

Free T4 Method

Total T4 Method

IS Addition
- $^{13}$C$_6$ T4
- Matched to approximate T4 concentration

Cleanup & Analysis
- SPE (C18)
- LLE (ethyl acetate)
- LC-MS/MS

CDC Method is aligned with the reference method established at the University of Ghent
CDC’s new reference methods meet or exceed performance requirements for reference methods

- Highly specific by use of isotope-dilution LC-MS/MS
- Traceable to SI: IRMM-468- certified, pure-compound reference materials as calibrators

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Total T4</th>
<th>Free T4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Accuracy</td>
<td>-0.5%</td>
<td>-0.2%</td>
</tr>
<tr>
<td>Precision</td>
<td>0.8% (inter-day)</td>
<td>1.1% (inter-day)</td>
</tr>
<tr>
<td></td>
<td>0.9% (intra-day)</td>
<td>2.7% (intra-day)</td>
</tr>
<tr>
<td>Analytical Measurement Range</td>
<td>1-20 mg/dL</td>
<td>0.3-8.0 ng/dL</td>
</tr>
</tbody>
</table>
CDC’s new reference method for FT4 is in good agreement with other reference labs

- Blinded euthyroid samples (20) measured by 4 reference labs show good correlation

- The CDC mean bias to the overall laboratory mean is within ±2.5% bias, and the bias is consistent across the 14–24 pmol/L concentration range
Next Steps

PTH
- Assess alternative sample preparation approaches to improve method sensitivity
- Measure additional PTH-related variants

Free T4
- Complete 2nd comparison with reference network using hypo/hyper/euthyroid samples
- Create reference materials for a thyroid hormone certification program to be launched in 2019

TSH
- Develop a harmonization program
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- Committee for the Standardization of Thyroid Function Tests

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THANK YOU

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Or visit https://www.cdc.gov/nceh/dls/ccb.html