Vitamin D Deficiency and Determination of 25-Hydroxyvitamin D by LC-MS/MS

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Vitamin D

- Metabolism and biological actions
- Recommended blood levels
- Ergocalciferol (D<sub>2</sub>) vs. cholecalciferol (D<sub>3</sub>)
- Methods for measuring vitamin D
- **25OHD by LC-MS/MS**
  - Extraction and isotopic dilution
  - Chromatographic separation
  - Detection by mass spectrometer
  - Data reduction and reporting
Vitamin D Metabolism

Food (Dietary Vitamin D) → Plasma Vitamin D₂, D₃ → Skin

Skin → 7-OH- calciferol

25 OH D₃ → Decreased PTH, Increased P, Ca

24,25 (OH)₂D₃ → Increased PTH, Decreased P, Ca

1,25 (OH)₂D₃ (Active Compound)
Is Vitamin D a Vitamin?

A compound is called a vitamin when it cannot be synthesized by an organism and must be obtained from the diet.

Vitamin D is not a vitamin
Biological Actions of Vitamin D

- Classical
  - Intestine: stimulate calcium absorption
  - Bone: bone formation and calcium mobilization
  - Kidney: mediate renal tubular phosphate and calcium reabsorption

- Non-classical
  - Dozens of target tissues
  - Anti-proliferative activity
  - Cell differentiation activity
  - Immune system modulation
Calcium Homeostasis

- Increases excretion of phosphate
Message # 1

Hormone: a complex chemical substance produced in one part or organ of the body that initiates or regulates the activity of an organ or a group of cells in another part of the body.

Vitamin D is a hormone!
Levels of Vitamin D Metabolites

<table>
<thead>
<tr>
<th>Compound</th>
<th>ng/mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vitamin D</td>
<td>0.2 – 20.0</td>
</tr>
<tr>
<td>25OHD</td>
<td>10 – 100</td>
</tr>
<tr>
<td>24,25(OH)₂D</td>
<td>2 – 5</td>
</tr>
<tr>
<td>1,25(OH)₂D</td>
<td>0.015 – 0.060</td>
</tr>
</tbody>
</table>

- ~ 40 minor metabolites of vitamin D
- 25OHD: best indicator of vitamin D status

**What is a “healthy” 25OHD level?**
### What Is Recommended Serum 25OHD Level?

<table>
<thead>
<tr>
<th>Source</th>
<th>ng/mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>From population-based study</td>
<td>9 – 54</td>
</tr>
<tr>
<td>Current reference range</td>
<td>30 – 100</td>
</tr>
<tr>
<td>Recent literature</td>
<td>&gt;32</td>
</tr>
<tr>
<td>Vitamin D council</td>
<td>35 – 55</td>
</tr>
<tr>
<td>IOM</td>
<td>&gt;20</td>
</tr>
</tbody>
</table>

*Vitamin D varies greatly depending on both season and latitude (sunlight).*
Vitamin D Daily Results Distribution

Data from Quest Diagnostics Nichols Institute
PTH Suppression Test

# 25OHD – What is “normal”?

<table>
<thead>
<tr>
<th>Parameter</th>
<th>25OHD (ng/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PTH suppression</td>
<td>&gt;30</td>
</tr>
<tr>
<td>Bone mineral density</td>
<td>36-40</td>
</tr>
<tr>
<td>Fracture prevention</td>
<td>36-40</td>
</tr>
<tr>
<td>Leg function</td>
<td>36-40</td>
</tr>
<tr>
<td>Periodontal disease</td>
<td>36-40</td>
</tr>
<tr>
<td>Colorectal cancer</td>
<td>36-40</td>
</tr>
</tbody>
</table>

These data suggest that the physiological lower limit of normal for 25OHD is ~35 ng/mL. The upper limit has not been determined but it is probably >100 ng/mL.
Messages #2

\[ \sim 45\% \text{ of U.S. population suffer from vitamin D deficiency and/or insufficiency} \]
Causes of Vitamin D Deficiency

Vitamin D deficiency is a disease of the industrial revolution (air pollution) and the use of clothes and sunscreen.
Vitamin D deficiency is the second most common endocrine disorder in this country – next to thyroid disorder
### Diseases Associated with Vitamin D Deficiency in Humans

- Osteoporosis and rickets
- Infectious disease
- Autoimmune diseases
  - Type 1 diabetes
  - Rheumatoid arthritis
  - Multiple sclerosis

- Cancers
  - Breast
  - Colon
  - Ovarian
  - Pancreas
  - Prostate

- Hypertension, cardiovascular disease
Cumulative Probability of 1st CV Event

Participants with hypertension

25 OHD <15 ng/mL
25 OHD ≥15 ng/mL

Participants w/o hypertension

25 OHD <15 ng/mL
25 OHD ≥15 ng/mL

Vitamin D Test Volume (2000-2008)

Medicare Part B Claims for Vitamin D Testing (000s)*

*Submitted Medicare claims for CPT 82306 (25-OH Vitamin D)
Source: CodeMap LLC and Laboratory Economics' estimate for 2008

Laboratory Economics 2009;4(1):4
# Human Sources of Vitamin D

<table>
<thead>
<tr>
<th></th>
<th>Cholecalciferol (Vitamin D₃)</th>
<th>Ergocalciferol (Vitamin D₂)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Sunlight (IU/d)</strong>*</td>
<td>2,000 – 20,000</td>
<td></td>
</tr>
<tr>
<td><strong>Diet (IU)</strong>†</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vitamins</td>
<td>200 – 1,000</td>
<td>200 – 1,000</td>
</tr>
<tr>
<td>Milk</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>Fish</td>
<td>200 – 400</td>
<td></td>
</tr>
<tr>
<td>Drisdol®</td>
<td>50,000</td>
<td></td>
</tr>
<tr>
<td>Mushrooms</td>
<td>500</td>
<td></td>
</tr>
</tbody>
</table>

*100 IU/day of vitamin D₃ ~ 25OHD increase of 1 ng/mL.
†1 international unit (IU) = 65 pmol = 25 ng.
Treatment of Vitamin D Deficiency

Drisdol® Tablets

- Only high-dose, prescription form of Vit D in U.S.
- 50,000 IU (1.25 mg) of ergocalciferol (Vit D₂)

U.S. promotes the pharmacological use of ergocalciferol (Vitamin D₂)

Elsewhere, high-dose forms of vitamin D are formulated with cholecalciferol (Vitamin D₃)
# How Much?

## Recommended dietary allowances (RDAs)

<table>
<thead>
<tr>
<th>Age</th>
<th>RDA (IU)</th>
<th>Upper Limit (IU)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0-6 months</td>
<td>400</td>
<td>1,000</td>
</tr>
<tr>
<td>6-12 months</td>
<td>400</td>
<td>1,500</td>
</tr>
<tr>
<td>1-3 years</td>
<td>600</td>
<td>2,500</td>
</tr>
<tr>
<td>4-8 years</td>
<td>600</td>
<td>3,000</td>
</tr>
<tr>
<td>9-70 years</td>
<td>600</td>
<td>4,000</td>
</tr>
<tr>
<td>&gt;70 years</td>
<td>800</td>
<td>4,000</td>
</tr>
</tbody>
</table>
How Much?

- Meta-analysis of randomized control trials (>80,000 fractures total)
- No reduction in fractures when vitamin D dose <400 IU/d
- Higher dose (482-770 IU/d) vitamin D reduced non-vertebral and hip fractures by more than 15%

How Much?

Testing is Necessary! (if you want to know how to treat the problem)

- At 1000 IU/day, the serum levels range from 15 ng/ml to about 85 ng/ml (38-212 nmol/L)
- 6000 IU/day would get 98% of the group above 40 ng/ml (100 nmol/L)
- At 10,000 IU/day, no one was above 200 ng/ml (500 nmol/L) (A level considered to anticipate toxicity)
- The red line shows a potential 'flattening' at higher levels
- 1014 people's data are in this chart

www.grassrootshealth.com, 05/10/2010
Levels of 25OHD Associated with Disease Prevention

<table>
<thead>
<tr>
<th>Disease Incidence Prevention by Serum 25(OH)D Level</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum 25(OH)D, ng/ml</td>
</tr>
<tr>
<td>----------------------</td>
</tr>
<tr>
<td>Studies of Individuals</td>
</tr>
<tr>
<td>Cancers, all combined</td>
</tr>
<tr>
<td>Breast Cancer</td>
</tr>
<tr>
<td>Ovarian Cancer</td>
</tr>
<tr>
<td>Colon Cancer</td>
</tr>
<tr>
<td>Non-Hodgkins Lymphoma</td>
</tr>
<tr>
<td>Type 1 Diabetes</td>
</tr>
<tr>
<td>Fractures, all combined</td>
</tr>
<tr>
<td>Falls, women</td>
</tr>
<tr>
<td>Natural Experiments</td>
</tr>
<tr>
<td>Kidney Cancer</td>
</tr>
<tr>
<td>Endometrial Cancer</td>
</tr>
<tr>
<td>Rickets</td>
</tr>
</tbody>
</table>

www.grassrootshealth.com, 07/26/2008
Cholecalciferol and Ergocalciferol

Humans make vitamin D₃ in the skin
Plants and yeast make vitamin D₂

25-Hydroxyvitamin D₃  
25-Hydroxycholecalciferol  
25OHD₃

25-Hydroxyvitamin D₂  
25-Hydroxyergocalciferol  
25OHD₂

400 daltons  
412 daltons
Messages #4

\[ D_2 > D_3 \]

\[ D_2 = D_3 \]

or

\[ D_2 < D_3 \]
LC/MS/MS Data on 25(OH)D vs Plateau Phase of iPTH Suppression

Salameh et al. *Endocrine Reviews*. 2010;31(3)[Suppl 1]:S238.
### Why Measure Both D₂ and D₃?

56 year-old female at risk for osteoporosis

<table>
<thead>
<tr>
<th></th>
<th>Baseline ng/mL</th>
<th>Post 12 wk Drisdol&lt;sup&gt;a&lt;/sup&gt; ng/mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>25OHD, Total</td>
<td>43</td>
<td>46</td>
</tr>
</tbody>
</table>

<sup>a</sup> 50,000 units once weekly.
Why?

- Lab error
- Patient non-compliance
- Seasonal change
- Doctor ordered wrong test
- All of above
Why Measure Both D$_2$ and D$_3$?

56 year-old female at risk for osteoporosis

<table>
<thead>
<tr>
<th></th>
<th>Baseline (ng/mL)</th>
<th>Post 12 wk Drisdol (ng/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>25OHD$_3$, Total</td>
<td>43</td>
<td>46</td>
</tr>
<tr>
<td>25OHD$_3$</td>
<td>27</td>
<td>10</td>
</tr>
<tr>
<td>25OHD$_2$</td>
<td>16</td>
<td>36</td>
</tr>
</tbody>
</table>

$^a$ 50,000 units once weekly.
25OHD LC-MS/MS Method

- 2 Simultaneous assays
  - Assay #1: 25OHD$_2$
  - Assay #2: 25OHD$_3$

- Procedure
  - Extraction and isotope dilution via protein precipitation
  - HPLC (high performance liquid chromatography)
  - MS/MS (tandem mass spectrometry)
  - Data reduction and reporting
**25OHD (LC-MS/MS) Step by Step**

**START PROCESS**
- Pickup specimens, set up worksheet, thaw specimens
- Place specimens in JANUS racks
- JANUS pipettes specimens to microtiter plates
- Microtiter plates vortexed (10 seconds each plate)
- Microtiter plates removed from centrifuge
- Microtiter plates loaded into MSMS, Start instrument

**END PROCESS**
- Verify and release results
- Microtiter plates centrifuged

**Step**: Where "n" is the number of times the step is repeated.

- Pincher motion
- Rotation
- Vibration
- Manual pipette

**NO MANUAL PI PETTING! NO ROTATIONAL MOVEMENTS! VIBRATIONAL STRESSORS REDUCED! 12 STEPS REMOVED!**
LC-MS/MS System

- Liquid chromatography, tandem mass spectrometry (LC-MS/MS)
- Throughput: ~1,000 cases/d
Chromatographic Separation

Chromatographic Process

B+A Enter the column

Mobile phase

Distribution:

\[ K = \frac{C_v}{C_{1u}} \]

Detector

Chromatogram

Dr. Shula Levin,
http://www.forumseil.co.il/hplc

Chromatography_process2.AVI
Basics of Mass Spectrometry

Sample Inlet

Ionization & Absorption of Excess Energy

Fragmentation (Dissociation)

Mass Analysis

Detection

m/z
MS/MS

Hydrocodone

300 m/z → 300 m/z → 257 m/z, 243

Codeine

300 m/z → 300 m/z → 282 m/z, 243, 215
Sample Preparation

- LC often obviates need for derivatization
- LC compatible with on-line extraction
- LC compatible with direct injection
Ionization Mechanisms

- Electrospray Ionization
- Atmospheric Pressure Chemical Ionization

2002 Nobel prize

"for their development of soft desorption ionization methods for mass spectrometric analyses of biological macromolecules"

John Fenn
Koichi Tanaka
Electrospray Ionization

Drying Gas

ESI needle

To mass spec

Capillary inlet
Mass Analyzers

- Linear quadrupole
- Ion traps
- **Triple quadrupole**
- Linear quadrupole ion traps
- Time of flight
- QTOF
- Orbitrap
Quadrupole Mass Analyzer

2006 Instrument Manual; courtesy of Thermo Electron Corporation
Animation of ion movement because of IE
MRM (Multiple Reaction Monitoring)

<table>
<thead>
<tr>
<th></th>
<th>Q1</th>
<th>Q3</th>
<th>Dwell time</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>453</td>
<td>254</td>
<td>20ms</td>
</tr>
<tr>
<td>2</td>
<td>685</td>
<td>885</td>
<td>20ms</td>
</tr>
<tr>
<td>3</td>
<td>453</td>
<td>254</td>
<td>20ms</td>
</tr>
<tr>
<td>4</td>
<td>396</td>
<td>274</td>
<td>20ms</td>
</tr>
<tr>
<td>5</td>
<td>1098</td>
<td>870</td>
<td>20ms</td>
</tr>
<tr>
<td>6</td>
<td>464</td>
<td>222</td>
<td>20ms</td>
</tr>
<tr>
<td>7</td>
<td>987</td>
<td>274</td>
<td>20ms</td>
</tr>
<tr>
<td>8</td>
<td>887</td>
<td>870</td>
<td>20ms</td>
</tr>
</tbody>
</table>
Tandem Mass Spectrometry

- Triple quadrupole mass spectrometer
- 3 quadrupoles
- Q1 = mass filter (for precursor ions)
- Q2 = collision cell
- Q3 = mass filter (for product ions)

2006 Instrument Manual; courtesy of Thermo Electron Corporation
Tandem MS - Cutaway View

2006 Instrument Manual; courtesy of Thermo Electron Corporation
Triple Quadrupole
LC-MS/MS Chromatographs

Blank
0 ng/mL 25OHD$_2$
0 ng/mL 25OHD$_3$

High Standard
128 ng/mL 25OHD$_2$
128 ng/mL 25OHD$_3$

Data from Quest Diagnostics Nichols Institute
LC-MS/MS Chromatographs

“Normal” patient
35 ng/mL 25OHD₃
<4 ng/mL 25OHD₂

Patient taking vitamin D₂
<4 ng/mL 25OHD₃
85 ng/mL 25OHD₂

Data from Quest Diagnostics Nichols Institute
LC-MS/MS Standard Curves

6-point standard curve: (128, 64, 32, 16, 8, 4 ng/mL)

Data from Quest Diagnostics Nichols Institute
## 25OHD LC-MS/MS Validation

### Reproducibility (%)

<table>
<thead>
<tr>
<th></th>
<th>Low (20-25 ng/mL)</th>
<th>Medium (45-55 ng/mL)</th>
<th>High (95-105 ng/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Intra-assay</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>25OHD$_2$</td>
<td>9.0</td>
<td>6.9</td>
<td>8.1</td>
</tr>
<tr>
<td>25OHD$_3$</td>
<td>11.2</td>
<td>7.5</td>
<td>8.5</td>
</tr>
<tr>
<td><strong>Interassay</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>25OHD$_2$</td>
<td>9.7</td>
<td>11.6</td>
<td>8.8</td>
</tr>
<tr>
<td>25OHD$_3$</td>
<td>13.5</td>
<td>10.7</td>
<td>8.4</td>
</tr>
</tbody>
</table>

Data from Quest Diagnostics Nichols Institute
25OHD LC-MS/MS Validation

Sensitivity (limit of quantitation)

Data from Quest Diagnostics Nichols Institute
## 25OHD LC-MS/MS Validation

### Specificity

<table>
<thead>
<tr>
<th>Compound</th>
<th>Compound Mass (Da)</th>
<th>Cross-reactivity (25OHD2)</th>
<th>Cross-reactivity (25OHD3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>25OHD2</td>
<td>412</td>
<td>100%</td>
<td>ND</td>
</tr>
<tr>
<td>25OHD3</td>
<td>400</td>
<td>ND</td>
<td>100%</td>
</tr>
<tr>
<td>6D-25OHD3</td>
<td>406</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Vitamin D2</td>
<td>396</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Vitamin D3</td>
<td>384</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>1α,25(OH)2D2</td>
<td>428</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>1α,25(OH)2D3</td>
<td>416</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>25,26(OH)2D3</td>
<td>416</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>1α(OH)D2</td>
<td>412</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>1α(OH)D3</td>
<td>400</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

Data from Quest Diagnostics Nichols Institute

ND, not detected
LC-MS/MS vs. HPLC

LC-MS/MS vs. V#1 RIA

LC-MS/MS vs. V#1 EIA

LC-MS/MS vs. V#2

LC-MS/MS vs. V#3 - Version 2

LC-MS/MS vs. V#4

LC-MS/MS vs. IAs
LC-MS/MS Advantages

- Automation – low hands-on-time
- Cost reduction – little reagent cost
- Multiplex – excellent efficiency
- Reproducibility – excellent precision/low CV
- Sensitivity – better than many of the IAs
- Small sample volume – few QNS
- Specificity – no immuno cross reactivity
- Versatility – large and small molecules
- Wide dynamic range – few repeats
Summary

- Vitamin D deficiency (<20 or <30 ng/mL total 25OHD) is very common
- Two different forms of 25OHD (D₂ and D₃) – both need to be measured to obtain total 25OHD concentration
- Many causes for vitamin D deficiency
- 25OHD (instead of 1,25 DHVD) is the marker for measuring vitamin D storage status
- Assay standardization is essential (population-based reference ranges are not recommended)
Summary

- Many implications for 25OHD deficiency
  - Conventional
  - Non-conventional
- Possible indicator/use as a chemo-preventive agent for various diseases/cancers
- Methods for vitamin D determination
- LC-MS/MS should be the method of choice