The Measurement of Vitamin D

“Solving a challenging assay in the clinical research laboratory”
Outline

- Introduction to Vitamin D
- Automated SPE approach to Vit D analysis
Vitamin D Deficiency

- Worldwide, an estimated 1 billion people don’t get enough of “the sunshine vitamin”

- Hundreds of studies now link vitamin D deficiency with significantly higher rates of many forms of cancer, as well as heart disease, osteoporosis, multiple sclerosis and many other conditions and diseases
If shadow TALLER than you are tall, you CANNOT make vitamin D

(UV index = 3)
What is Vitamin D?

Major source – sunlight

Cholecalciferol (vitamin D₃)

Liver

25 hydroxyvitamin D₃

Kidney

1,25-dihydroxyvitamin D₃

↑Calcium absorption (small intestine)
↑Urinary calcium re-absorption (kidney)
↑Bone mineralisation

Maintains calcium balance in the body via the action of parathyroid hormone

Minor source – dietary intake

Vitamin D₃ (fish, meat)
Vitamin D₂ (vitamin supplements)
There are two forms of Vitamin D; Vitamin D₃ produced by the body and Vitamin D₂ from the diet.

- **Ergocalciferol**
  - Vitamin D₂
  - MW 396.7

- **Cholecalciferol**
  - Vitamin D₃
  - MW 384.6
25-OH vitamin D is the storage form of Vitamin D and therefore its concentration reflects vitamin D status.

25-OH vitamin D analysis therefore provides information relating to either Vitamin D deficiency, insufficiency or toxicity.

Why measure 25-OH vitamin D$_2$ as well?
- Common form of supplementation
- Measure to prevent toxicity and compliance
### Definitions of Vitamin D deficiency

<table>
<thead>
<tr>
<th>25(OH) Vitamin D level</th>
<th>ng/mL</th>
<th>nmol/L</th>
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<tbody>
<tr>
<td>Severe Deficiency</td>
<td>&lt;6</td>
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<td>Deficiency</td>
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<td>Insufficiency</td>
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<td>Normal</td>
<td>&gt;20</td>
<td>&gt;50</td>
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*Mosekilde L et al, Ugeskr Læger 167/1, 2005, 29-33*
Growing evidence suggests that chronically low levels of vitamin D raise a person’s risk for certain major illnesses based on blood serum levels, including:

- 30% of patients with prostate cancer
- Five times higher risk of lung cancer in high latitude Northern Europe living
- 77% lower risk of breast cancer
- 30% lower risk of multiple sclerosis at serum 25D levels above 40 ng/ml than at 25 ng/ml or less
- 80% lower lifetime risk for autoimmune (type 1) diabetes in Finnish children given 2,000 IU of D3 daily during first year of life

We recognize that vitamin D does much more than build bones and that many people are not getting enough of it. D D deficiency contributing to major illnesses?

Not Enough Sunshine? Vitamin D Fights Cancer

Vitamin D society.org
“Because of the varied ethnicities, latitudes and lifestyles in the United States and, most of Europe too, the only way to safely use vitamin D is test, test and retest.”

Increased demand for Vitamin D assays

- USA
  - 90% increase over last 12-24 months
  - > 4 Million Vitamin D Assays
- UK
  - requests increased from 4000 in 2005 to >400,000 per year in 2011
How are you currently measuring Vitamin D?

- Immunoassay or HPLC/UV?

- Do you know what you are measuring/reporting?
  - 25(OH)D$_3$ only, 25(OH)D$_3$ and 25(OH)D$_2$

- Can you be sure of this?
  - Immunoassays
    - Prone to interferences
    - Variable recovery of 25(OH)D$_2$
Why change to LC/MS/MS?

- **Analytical Performance**
  - LC/MS/MS is recognised as the gold standard
  - Measures both 25(OH) Vit D₃ and D₂
  - Increased specificity and selectivity from MRM and UPLC

- **Economics**
  - Reimbursement (US)
    - 25 OH Vit D $45.02
    - Immunoassay costs $10-$18 per sample
  - Instrument Payback in < 2 yrs
    - 10,000 samples per year by Immunoassay
    - $180,000 in reagents costs alone
The assay crying out for standardisation and harmonisation

When eight labs all running validated, routine clinical LC/MS/MS assays for 25(OH) Vit D were asked to analyse 16 patient pooled samples

- Different LC and MS manufacturers
- “Home-Brew”
- Some using commercial standards (Recipe from Munich)
Analysis of 25(OH) D₃: In house calibrators

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<tr>
<th>Pool Number</th>
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Analysis of 25(OH) Vit D3

- **Pool Number**: Represents the different samples or pools analyzed.
- **Conc (ng/mL)**: Concentration of 25(OH) D₃ in nanograms per milliliter for each pool analyzed by different laboratories (Lab 1 to Lab 8).
Analysis of 25(OH) Vit D3 with harmonised calibrators

Pool Number

Conc (ng/mL)

Lab 1
Lab 2
Lab 3
Lab 4
Lab 5
Lab 6
Lab 7
Lab 8
The need for standardisation and harmonisation

- Variation may explain the performance of MS in Proficiency Testing schemes
- Which lab is right?
- Is it important?
  - Establish reference ranges
- Standardisation
  - International Reference Standard
- Harmonisation
  - Common calibrators and sample preparation will allow harmonisation of results between different labs
Calibrators
- Preparation of in house calibrators is difficult
- Poor solubility of Vitamin D
- Stripped Serum, very difficult to obtain, batch to batch variation, cost

Choice of matrix affects the calculated levels of Vit D

Recipe supplies calibrators, controls and standards
Requirements of a Vitamin D assay

- Easy to use assay
- Individual measurement of 25(OH)Vitamin D$_2$ and D$_3$
- High Specificity
- Robust assay
- Sensitivity <5 ng/mL
- Fast analysis
- Automated
Automated off-line Solid-phase extraction of 25-hydroxyvitamin D from serum for measurement by UPLC/MS/MS
Method for the analysis of 25OHD2 and 25OHD3

Semi-automated using a Tecan Freedom EVO 100
  — High throughput

µElution SPE
  — Waters Oasis chemistry
  — No evaporation step required

Calibration range 2.5-150ng/mL (6-375nmol/L)
  — <6ng/mL is severely deficient 25OHD
  — >20ng/mL is sufficient 25OHD

Sub 5-minute run time
  — Aqueous methanol gradient
Waters Semi-Automated 25OHD SPE Method

- Semi-automated SPE sample preparation
- Barcode reader
  - Patient samples scanned as rack is loaded onto robot
  - Tracking of labware and reagents
- Te-VacS
  - Vacuum manifold allowing automation of SPE from plate equilibration through to sample elution

Tecan Freedom EVO 100
Waters Semi-Automated 25OHD SPE Method

- Sample preparation workflow
  - 96 samples prepared in less than 2hrs

- All liquid handling steps executed by the robot
  - eliminating operator error
  - minimising operator intervention

- Only the centrifugation step does not take place on the Tecan

Sample, labware and reagents tracking, connectivity to LIMS

1. Identify components
2. PPT proteins centrifugation
3. Condition equilibrate
4. Load supernatant wash
5. Elute
6. Transfer to UPLC
- Water loss transition used for qualifier but not quantifier
- Water loss transitions not as specific

<table>
<thead>
<tr>
<th>Compound</th>
<th>MRM transition (m/z)</th>
<th>Cone voltage (V)</th>
<th>Collision Energy (eV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>25OHD3</td>
<td>401.35&gt;159.1</td>
<td>26</td>
<td>28</td>
</tr>
<tr>
<td>25OHD3</td>
<td>401.35&gt;383.2</td>
<td>26</td>
<td>12</td>
</tr>
<tr>
<td>25OHD2</td>
<td>413.35&gt;355.2</td>
<td>26</td>
<td>10</td>
</tr>
<tr>
<td>25OHD2</td>
<td>413.35&gt;83.1</td>
<td>26</td>
<td>22</td>
</tr>
</tbody>
</table>
25OHD3

Compound name: 25OHD3
Correlation coefficient: $r = 0.9996$, $r^2 = 0.9993$
Calibration curve: $0.0286787 \times x + 0.0166297$
Curve type: Linear, Origin: Exclude, Weighting: 1/x, Axis trans: None
Intra- and inter-assay precision for 25OHD3 QCs (pooled human serum)

<table>
<thead>
<tr>
<th>Level</th>
<th>QC1</th>
<th>QC 2</th>
<th>QC 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>mean (ng/mL)</td>
<td>8</td>
<td>34</td>
<td>81</td>
</tr>
<tr>
<td>Intra-assay %CV</td>
<td>7.13</td>
<td>6.15</td>
<td>4.33</td>
</tr>
<tr>
<td>Inter-assay %CV</td>
<td>8.67</td>
<td>9.75</td>
<td>7.99</td>
</tr>
</tbody>
</table>

Analysis of DEQAS samples within ±10.8% of LC/MS method mean

Recovery: ~80% for both analytes

No carryover observed following 200ng/mL injection
Scatter Plot with Passing & Bablok Fit

**Waters**

**UHSM**

Identity

**Passing & Bablok (I) fit**

\(-1.45 + 1.01x\)

95% CI bands
Patient sample: low [25OHD3]

25OHD3 Internal Standard

Patient sample
[25OHD3] 6.9 ng/mL
Patient sample: low [25OHD2]

25OHD2 Internal Standard

MRM of 6 Channels ES+
416.25 > 358.25 IS (Vij D2)
2.84e4
Area

Pooled Serum
[25OHD2] 7.7ng/mL

MRM of 6 Channels ES+
413.25 > 355.25 (D2)
8.26e3
Area
Vitamin D Summary

- Developed a sensitive assay for 25OHD2 and 25OHD3 that requires very little operator intervention
- µElution SPE is attractive as it lends itself well to automation
- Semi-automated sample preparation could be a solution for your increased sample throughput
Sample preparation is required for endocrine applications
   — Protein precipitation, liquid-liquid or solid-phase extraction are all suitable for analysis of steroids by LC/MS/MS
   — As the test requests increase, automation of sample preparation is more attractive
     o minimal operator involvement

For a robust method
   — Ask Waters!
   — Other methods currently under investigation
     o Plasma mets
     o Urinary free cortisol
     o Aldosterone