LC/MS/MS measurement of Vitamin D3 and D2: Present and Future

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Overview

1. 25 hydroxy Vitamin D3 overview
2. Current assays and problems
3. Current LC MS/MS methodologies
4. New developments
5. Case history
1. 25 hydroxy Vitamin D3 overview
Major source – sunlight

Skin

Cholecalciferol (vitamin D₃)

Liver

25-dihydroxyvitamin 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
• Blood is the largest single pool of 25-OH D3 and the circulating concentration of 25-OH D3 is used as a measure of vitamin D status (should be maintained > 75 nmol/L)

• Intestinal calcium transport increased 45-65% when 25-OH Vit D3 levels increased from an average 50 to 80 nmol/L

• Using this definition, estimated the 1 billion people worldwide have Vitamin D deficiency
Classical functions of vitamin D:

- Regulation of calcium homeostasis and bone mineralization
- Promotes intestinal absorption of calcium
- Promotes resorption of Ca++ in kidneys
- Mobilizes Ca from bones thereby initiating bone remodeling process at the same time promotes Ca Po4 into rachitic and osteoporotic bones

Supplementary functions:

- Directly or indirectly controls more than 200 genes
- Helps to regulate immune system
- Regulates cell proliferation, differentiation and apoptosis
Non-skeletal Implications of Vitamin D3 deficiency

- colorectal cancer
- multiple sclerosis, rheumatoid arthritis
- type 1 diabetes
- blood pressure
- congestive heart failure
- mortality in CKD
Vitamin D deficiency is common

- Vitamin D deficiency: an emerging public health problem in Australia\(^1\) (all over the world)
- Deficiency → bone pain, muscle weakness, osteoporosis, falls, fractures\(^1\)
- 60% of postmenopausal Australian women with osteoporosis had low serum vitamin D (< 75 nmol/L)\(^2*\)

* International study of 2606 postmenopausal women with osteoporosis, including 204 women from Australia

1. Osteoporosis Australia. Calcium, Vitamin D and Osteoporosis – A Guide for GPs 2\(^{nd}\) edn
Early symptoms of vitamin D deficiency (Osteomalacia)

- Muscle pain mainly shoulder /hip girdle
- Recurrent falls and difficulty transferring in elderly
- Recurrent fractures
- Poor fracture healing
- Bone pain
  - particularly with bisphosphonates
- Premature OA

Mayo clinic proceedings Dec 2003  Plotnikoff GA Quicgley JM
Prabhala A Arch Intern Med 2000
Al Faraj et al Spine 2003
Pfeifer M et al J Bone Miner 2000
M.Hollick Vit D Millinium Perspective J Cell Biochem 2003
Factors affecting Vitamin D production on skin

- Season
- Geographic latitude
- Time of day
- Cloud /fog
- Sun screen
- Ageing skin
- Excess skin cover
- Window glass
- Indoor life style
Who may need extra Vitamin D?

- Infants who are exclusively breast fed
- Older adults
- Persons with limited sun exposure
- People with pigmented skin
- Patients with malabsorption
- Patients on prednisolone & thyroid supplements and those on antiepileptic

Dietary supplements Fact Sheet Vit D National Inst. Of Health
Vitamin D is a Hormone or a Vitamin?

- Vitamin D fits the definition of a Vitamin and that of a Hormone
Vitamin D: A Hormone & A Vitamin

HORMONE
• A messenger produced and secreted by specific glands or cells within the body of animals.
• Transported through the blood stream to designated target organs.
• Binds to its specific receptor delivering its message to a specific set of cells.

VITAMIN
• A substance regularly required by the body in small amounts.
• The body cannot make vitamins.
• Must be supplied in diet.
2. Current Assays and problems
ISSUES

• $D_3$ – cutaneously derived
• $D_2$ – food supplements
• $D_2 < D_3$ potentency
• $D_3 > D_2$ duration of action
• $25(OH)D$ circulates bound to Vit D BP
• Genetic variants of Vit D BP
• Interference of serum matrix factors
• Various assay techniques
• I As - $25(OH)D_2 \neq 25(OH)D_3$

All impact on the assessment of Vit D status
Accuracy and clinical implications of seven 25-hydroxyvitamin D methods compared with liquid chromatography–tandem mass spectrometry as a reference

Heinz Jurgen Roth, Heinrich Schmidt-Gayk, Holger Weber and Christoph Niederau

Limbach Laboratory, Department of Endocrinology and Oncology, Im Breitspiel 15, 69126 Heidelberg, Germany

Annals of Clin Biochem 2008; 45: 153-159
## Method Characteristics

<table>
<thead>
<tr>
<th>Method</th>
<th>BP Inact</th>
<th>Ab/BP</th>
<th>Xreact</th>
<th>CV%</th>
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<tr>
<td>LC-MS/MS</td>
<td>acetonitrile</td>
<td>-</td>
<td>nil</td>
<td>5.1(13), 3.2(48)</td>
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<td>HPLC</td>
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<td>-</td>
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<td>6.5(73), 2.3(250)</td>
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<tr>
<td>IDS-RIA</td>
<td>NaOH / acetonitrile</td>
<td>polyclon sheep</td>
<td>D₂ 75%</td>
<td>8.1(58), 7.3(135)</td>
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<td>IDS-enz</td>
<td>proprietary buffer</td>
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<td>D₂ 75%</td>
<td>6.4(73), 8.7(133)</td>
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<td>proprietary buffer</td>
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<td>D₂ 100%</td>
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<tr>
<td>Elecsys</td>
<td>polyclon sheep</td>
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<td></td>
<td>4.7(48), 5.1(178)</td>
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Regression Analysis vs LC-MS/MS
( Passing-Bablok)

<table>
<thead>
<tr>
<th>Method</th>
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<th>intercept</th>
<th>slope</th>
<th>r</th>
</tr>
</thead>
<tbody>
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<td>LIAISON</td>
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<td>4.3</td>
<td>0.83</td>
<td>0.95</td>
</tr>
<tr>
<td>Elecsys</td>
<td>291</td>
<td>-3.4</td>
<td>0.94</td>
<td>0.93</td>
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</table>
# Data Comparison ( >50% 25 OH D₂)

<table>
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<tr>
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<th>intercept</th>
<th>slope</th>
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</tr>
</thead>
<tbody>
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<td>7.9</td>
<td>0.84</td>
<td>0.95</td>
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<td>IDS-RIA</td>
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<td>-15</td>
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<td>0.96</td>
</tr>
<tr>
<td>E170</td>
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<td>2.8</td>
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<td>E170</td>
<td>27</td>
<td>-8.3</td>
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<td>LIAISON</td>
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<td>6.2</td>
<td>0.82</td>
<td>0.86</td>
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<td>LIAISON</td>
<td>30</td>
<td>33</td>
<td>1.76</td>
<td>0.95</td>
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</tbody>
</table>

3. Current LC MS/MS methodologies
# 25-OH Vitamin D Workload

<table>
<thead>
<tr>
<th>Year</th>
<th>Tests / yr</th>
<th>Tests / day</th>
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<tbody>
<tr>
<td>2006</td>
<td>8573</td>
<td>33</td>
</tr>
<tr>
<td>2007</td>
<td>13667</td>
<td>52</td>
</tr>
<tr>
<td>2008</td>
<td>16993</td>
<td>65</td>
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<tr>
<td>2009</td>
<td>32510</td>
<td>125</td>
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</tbody>
</table>

Turn-around time and service delivery contracts
Current Sample preparation procedures

• Liquid Liquid Extraction (LLE) using hexane
  – Manual extraction with several laborious steps (transfer of hexane layer, drying down and reconstitution in MP and transfer to vials)
  – Limited number of samples can be prepared per day (150)
  – Significant amount of waste generated ie glass tubes, transfer pipettes and solvents
  – Ion suppression issues and variable recovery
  – Very good precision and accuracy
Problems to overcome to increase throughput

- Worklist generation and sample alignment
- Transfer of organic layer
- Dry down
- Reconstitution in MP
- Transfer to vial
- Ion suppression issues
4. New developments
Automation

• Move to SPE to allow improved sample cleanup
• Minimise extraction steps to ones that can be automated
• Less waste generation
• Aim to elute and shoot
SPE format

- Reverse phase packing bed SPE
- 96 well format
- Small bed weight ie <20mg
  - Low volume washes
  - Elute in a small volume, minimise dilution effect
  - Inject elution solvent: NB must be fully compatible with chromatography MP and not affect chromatographic peak shape or resolution
Chromatography

- System must be able to cope with number of samples ie extract 300 sample/day and run
- UPLC allows quick chromatography time (4 min cycle time) including a gradient
- Theoretical sample throughput 360 samples/day
- Column life (Acquity BEH C8 2.1 x 50 mm 1.7u) with an in-line filter approximately 7000 injections
Vit D3 extraction protocol

- 150 ul plasma + internal Std (d4 Vit D3) + 150 ul 0.2 M Zinc Sulphate
- Vortex and add 500 ul methanol
- Vortex and centrifuge
- Place on activated 10mg OASIS HLB SPE column
- Wash with 60% methanol
- Elute with 100 ul methanol/IPA (80/20)
- Elute with 50 ul water
- Final organic conc matches initial MP conditions
## Inter-run imprecision results for Vitamin D2 and D3

<table>
<thead>
<tr>
<th>Analyte</th>
<th>MEAN</th>
<th>SD</th>
<th>CV%</th>
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</thead>
<tbody>
<tr>
<td>Vitamin D2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Chromsystems QC1</td>
<td>42.4</td>
<td>2.5</td>
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<td></td>
<td>Chromsystems QC 2</td>
<td>91.1</td>
<td>6.0</td>
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<td></td>
<td>UTAK Low</td>
<td>10.0</td>
<td>0.8</td>
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<tr>
<td></td>
<td>UTAK QC1</td>
<td>60.0</td>
<td>4.2</td>
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<tr>
<td></td>
<td>UTAK QC2</td>
<td>146.7</td>
<td>8.8</td>
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<tr>
<td>Vitamin D3</td>
<td></td>
<td></td>
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<tr>
<td></td>
<td>Chromsystems QC1</td>
<td>76.4</td>
<td>3.9</td>
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<tr>
<td></td>
<td>Chromsystems QC 2</td>
<td>180.7</td>
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<td>26.5</td>
<td>1.4</td>
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<td>UTAK QC1</td>
<td>72.7</td>
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<tr>
<td></td>
<td>UTAK QC2</td>
<td>194.6</td>
<td>10.0</td>
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</tbody>
</table>
Liquid chromatography solvent gradient for the UPLC MS/MS method.

<table>
<thead>
<tr>
<th>TIME (mins)</th>
<th>FLOW RATE (mL/min)</th>
<th>A</th>
<th>B</th>
<th>CURVE</th>
</tr>
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<tbody>
<tr>
<td>0</td>
<td>0.4</td>
<td>27.0</td>
<td>73.0</td>
<td>1</td>
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<td>1.5</td>
<td>0.4</td>
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<tr>
<td>3.0</td>
<td>0.4</td>
<td>2.0</td>
<td>98.0</td>
<td>6</td>
</tr>
<tr>
<td>3.5</td>
<td>0.4</td>
<td>2.0</td>
<td>98.0</td>
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<tr>
<td>3.6</td>
<td>0.4</td>
<td>27.0</td>
<td>73.0</td>
<td>6</td>
</tr>
</tbody>
</table>

Time measured in minutes, Curve 6 refers to a linear change between initial and final conditions
A: 2 mmol/L ammonium acetate in water with 0.1% formic acid
B: 2 mmol/L ammonium acetate in methanol with 0.1% formic acid
## Transitions

<table>
<thead>
<tr>
<th>COMPOUND</th>
<th>MRM FUNCTION</th>
<th>DWELL (Sec)</th>
<th>CONE (V)</th>
<th>COLLISION ENERGY (eV)</th>
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</thead>
<tbody>
<tr>
<td>d6-Vitamin D3 Quantifier</td>
<td>407.35&gt;159.10</td>
<td>0.100</td>
<td>23</td>
<td>25</td>
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<tr>
<td>d6-Vitamin D3 Qualifier</td>
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<td>0.050</td>
<td>23</td>
<td>9</td>
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<tr>
<td>25(OH) Vitamin D3 Quantifier</td>
<td>401.35&gt;159.10</td>
<td>0.100</td>
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<td>25</td>
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<td>25(OH) Vitamin D3 Qualifier</td>
<td>401.35&gt;383.35</td>
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<td>25(OH) Vitamin D2 Quantifier</td>
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<td>0.050</td>
<td>24</td>
<td>9</td>
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</tbody>
</table>
5. Case History
Male 22 years

- 22yr African American male with developmental delay
- Presented with possible seizure activity
- Serum Chemistry
  - Calcium 1.30 mmol/L
  - Phosphate 0.65 mmol/L
  - 25-OH Vit D3 <10 nmol/L
- Hypocalcaemia secondary to Vit D Deficiency
Followup

- Mother disclosed that son had not left home for last 3 months
- Previously involved in a day program
- Increased irritability over this time
- Limited diet (Vit D poor foods)
- Limited outdoor activity and restricted diet likely causes of Vit D deficiency
- Patient began Vit D replacement 500 000 IU daily and intravenous calcium
- Significant improvement and discharged in 4 days, more interactive and content than last 3 months. No more seizures or fits
Male 70 years

- Active outdoor lifestyle
- Type 2 diabetic
- Lipitor for 5 years
- Complaining of back and muscle pain (myalgia)
- Endocrinologist suggested Vit D3 status assessment
• 25 OH Vitamin D3  32 nmol/L (> 75 nmol/L)
• Treated with bolus 200 000 IU Vit D3
• Interaction between Lipitor and Vitamin D?
• Several recent publications noted a relationship between the statins and vitamin D status whereas some studies indicate the absence of a relationship