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Technical Aspects in the Measurement of Vitamin D Status

Talk Overview:

• What should we be measuring: 25-OH-D or 1,25-(OH)₂D?

• Brief overview of current methods
  - Antibody-based Methods
  - LC-based Methods

• Current controversies in vitamin D assay
  - What is the Normal range for 25-OH-D?
  - Performance Characteristics of Current 25-OH-D Assay
  - Standard Reference Material
  - Are Vitamin D₂ and Vitamin D₃ biologically equivalent?
  - Frequency of 25-OH-D assay?
Metabolism of Vitamin D$_3$

Vitamin D$_3$ → 25-OH-D$_3$ → 1α,25-(OH)$_2$D$_3$

CYP27A1, CYP2R1: Liver
CYP2B1: Kidney

Calcitriol

Similar pathway exists for vitamin D$_2$
Metabolism of Vitamins D₃ and D₂

Vitamin D₃

- CYP27A1
- CYP2R1
- Liver

25-OH-D₃

- CYP27B1
- Kidney

1α,25-(OH)₂D₃

Vitamin D₂

- CYP2R1
- CYP3A4
- Liver

25-OH-D₂

- CYP27B1
- Kidney

1α,25-(OH)₂D₂
DiaSorin RIA, $^{125}$I-ligand
- ACN extraction, primary & secondary Ab
- Co-specific for 25-OH-D$_2$ and 25-OH-D$_3$

IDS RIA, $^{125}$I-ligand
- ACN extraction, primary & secondary Ab
- Discriminates against 25-OH-D$_2$ (0.75)

DiaSorin Liaison, chemiluminescence
- Whole serum, w/ antibody coated particles
- Detects 25-OH-D$_2$ & 25-OH-D$_3$, 180 smpls/h

IDS EIA on New Dedicated Instrument
- No extraction, biotin labeled ligand
- Avidin-labeled horse radish peroxidase
- Discriminates against 25-OH-D$_2$ (0.75)

Roche E170 Analyzer
- Automated electro-chemiluminescence method
- Detects ONLY 25-OH-D$_3$
Current Controversies with 25-OH-D assay

(a) **What is the normal range?**

(b) **Performance Characteristics of Current 25-OH-D Assays**
   i) Vitamin D External Quality Assessment Scheme (DEQAS)
   ii) Measurement of Total 25-OH-D in samples
   iii) Measurement of 25-OH-D$_2$ content
   iv) Pediatric samples

(c) **Are Vitamin D$_2$ and Vitamin D$_3$ biologically equivalent?**
   Are separate assays of 25-OH-D$_2$ and 25-OH-D$_3$ clinically useful?

(d) **Can we avoid use of 25-OH-D assay?**
   Suggested frequency of 25-OH-D Testing
Plasma 25-OH-D Ranges

Observed Normal ranges

Jones (1978)- HPLC Assay = 9.1 - 23.9 ng/mL (Winter)

Hollis (1997) - RIA Assay = 9.9 - 41.5 ng/mL 
(Chapter 38- ‘Vitamin D’ 1st Edition)

Hollis (2005)- CLIA Assay = 9.5 - 52.0 ng/mL
(Chapter 58 ‘Vitamin D’ 2nd Edition)

Proposed Target ranges

Deficiency
Insufficiency
Sufficiency
Toxicity

K/DOQI

Normal function (%)

5  30  40  100

ng/mL
Seasonal variance of 25-(OH) vitamin D in the general population of Estonia, a Northern European country at Latitude 59° N
Mart Kull Jr*1,2, Riina Kallikorm1,2, Anu Tamm2 and Margus Lember1,2

BMC Public Health 2009, 9:22
Prevention and Treatment of vitamin D insufficiency and vitamin D deficiency

SUGGESTED THRESHOLD = 30 ng/mL or 75 nmol/L

Vitamin D intoxication at 25-OH-D >250 ng/mL

Adapted from Hollis
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Experience in clinical chemistry laboratories suggests that participation in an external quality assessment scheme (EQAS) is a prerequisite for improved analytical performance. The 25 hydroxyvitamin D EQAS (DEQAS) was launched in 1989 after several surveys\textsuperscript{1,2} revealed serious inconsistencies among laboratories measuring the analyte. The scheme was expanded in 1997 to include 1,25 dihydroxyvitamin D.

The widespread use of commercial assays coupled with the need for accreditation has stimulated considerable interest in DEQAS which has over 180 participants in 23 countries.

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An International Programme for monitoring the accuracy and precision of 25 Hydroxyvitamin D and 1,25 Dihydroxyvitamin D Assays

ASSAY METHOD PERFORMANCE

LC-BASED versus ANTIBODY-BASED DIFFERENT STRENGTHS & WEAKNESSES

OPERATOR PERFORMANCE

LEVEL OF EXPERIENCE WITH VITAMIN D
What is the ‘Gold Standard’ to judge 25-OH-D Assays by?

1) **Gas Chromatography-Mass Spectrometry (GC-MS)**

Extraction; lengthy purification; derivatization; GC; detection of fragments


2) **HPLC with UV Detection**

Extraction; clean-up on LC-1; separation 25-OH-D$_2$ & 25-OH-D$_3$ on LC-2; UV


3) **All-Laboratory Trimmed Mean (ALTM) from 650+ labs**

Adopted by DEQAS as appropriate tool for comparison of data in lieu of GC-MS

4) **Vitamin D Council claims it is the Diasorin RIA assay!**

John Cannell promotes the Diasorin assay as a check for the blood spot assay
FIGURE 4  Imprecision of 25-OHD results (all participants) from 1994 to 2009.*
FIGURE 5  Relative performance of 25-OHD methods in the last two distribution cycles.
Accuracy and clinical implications of seven 25-hydroxyvitamin D methods compared with liquid chromatography–tandem mass spectrometry as a reference

Heinz Jürgen Roth¹, Heinrich Schmidt-Gayk¹, Holger Weber² and Christoph Niederau³
COMPARISON OF HPLC vs DIASORIN RIA

n = 174
r = 0.7509
y = 0.709x - 5.86 µg/L
$S_{y|x} = 7.35$ µg/L
COMPARISON OF HPLC vs DIASORIN RIA

**25(OH)D₂**
- \( n = 18 \)
- \( r = 0.9965 \)
- \( y = 0.902x - 0.66 \mu g/L \)
- \( S_{yl|x} = 2.56 \mu g/L \)

**25(OH)D₃**
- \( n = 23 \)
- \( r = 0.9867 \)
- \( y = 1.01x - 4.82 \mu g/L \)
- \( S_{yl|x} = 4.93 \mu g/L \)
RECOVERY OF 25-OH-D$_2$–ENRICHED SAMPLES

![Bar chart showing recovery of 25-OH-D$_2$ in enriched samples using different methods. The x-axis represents the methods, and the y-axis represents the total 25-OH-D nmol/L. Method 1 and Method 3 show the highest recovery.]
LC-MS/MS analysis of 25-OH-D

25-OH-D<sub>3</sub>
- MS1
- 423[M+Na]<sup>+</sup>
- 401[M+H]<sup>+</sup>
- 383[M+H-H<sub>2</sub>O]<sup>+</sup>
- 439
- 455

25-OH-D<sub>3</sub>
- MRM
- 401→383
- Retention Time: 6.00 minutes

25-OH-D<sub>2</sub>
- MS1
- 435[M+Na]<sup>+</sup>
- 413[M+H]<sup>+</sup>
- 395[M+H-H<sub>2</sub>O]<sup>+</sup>
- 451
- 467

25-OH-D<sub>2</sub>
- MRM
- 413→395
- Retention Time: 6.29 minutes
C-3 Epimers Can Account for a Significant Proportion of Total Circulating 25-Hydroxyvitamin D in Infants, Complicating Accurate Measurement and Interpretation of Vitamin D Status


Departments of Laboratory Medicine and Pathology (R.J.S., R.L.T., S.K.G.G.) and Medicine (S.K.G.G.), Mayo Clinic, Rochester, Minnesota 55905; and Epimer, LLC (G.S.R.), Providence, Rhode Island 02906

Context: We have recently introduced liquid chromatography-tandem mass spectrometry (LC-MS/MS) for 25-hydroxyvitamin D$_2$ (25OHD$_2$) and 25OHD$_3$ testing. During subsequent clinical use, we identified significantly elevated results in some infants. We hypothesized this might represent assay interference caused by C-3 epimers of 25OHD$_2$ or 25OHD$_3$.

Objective: Our aims were to 1) determine the prevalence of C-3 epimers of 25OHD$_2$ or 25OHD$_3$ in human serum, and 2) identify the patient populations that might be affected.

Study Design: We modified our LC-MS/MS method to allow detection of C-3 epimers. We retested specimens from four patient groups with the new method and an extracted RIA: 1) children less than 1 yr old, 2) children 1–18 yr old, 3) adults aged 20–87 yr with liver disease, and 4) adults aged 19–91 yr without liver disease.

Results: In 172 children from group 1 with detectable 25OHD$_2$ or 25OHD$_3$, we identified C-3 epimers in 39 (22.7%). The epimers contributed 8.7–61.1% of the total 25-OHD. There was an inverse relationship between patient age and epimer percentage ($r = 0.48$; $P < 0.002$). The RIA gave accurate 25-OHD results that correlated with the modified LC-MS/MS method. No C-3 epimers were detected in any of the other groups.

Conclusions: Significant concentrations of C-3 epimers of 25OHD$_2$ or 25OHD$_3$ are commonly found in infants. This can lead to overestimation of 25-OHD levels. Measurements in children less than 1 yr should therefore be performed with an assay that allows accurate detection of 25-OHD in the presence of its C-3 epimers. (*J Clin Endocrinol Metab* 91: 3055–3061, 2006)
Comparison of 25-OH-D Structures

Mw: 400

25-OH-D₃

Mw: 400

3-epi-25-OH-D₃

Mw: 412

25-OH-D₂

Mw: 412

3-epi-25-OH-D₂
Development of SRM 972

Level 1
65 ± 15 nmol/L 25-hydroxyvitamin D₃ (“normal”)

Level 2
Blend of “normal” serum and horse serum to obtain approximately half the level of 25-hydroxyvitamin D₃ in the “normal” pool (35 ± 5 nmol/L)

Level 3
“Normal” serum spiked with an equivalent amount of 25-hydroxyvitamin D₂

Level 4
“Normal” serum spiked with 3-epi-25-hydroxyvitamin D₃

(Courtesy of Karen Phinney, NIST)
SRM 972 Vitamin D in Human Serum

- Four levels, each containing 1.0 mL serum
- Certified and reference values for 25(OH)D$_2$, 25(OH)D$_3$, and 3-epi-25(OH)D$_3$
- Value assignment by isotope-dilution LC-MS and LC-MS/MS using data from NIST and CDC
- Metabolite concentrations reported in ng/g, ng/mL, and nmol/L
- COA does not provide data from other analytical techniques

(Courtesy of Karen Phinney, NIST)
Development of Methodology

SRM 972 Level 1

- ID-LC/MS/MS and ID-LC/MS methods were developed
- Stable isotope labeled internal standards were utilized for measurements of 25(OH)D$_2$ and 25(OH)D$_3$
- ID-LC/MS/MS was validated for submission to JCTLM as a Reference Measurement Procedure
- 3-epi-25(OH)D$_3$ fully resolved from 25(OH)D$_3$; (separation based on the work of Lensmeyer et al.)

(Courtesy of Karen Phinney, NIST)
Impact

Sales have greatly exceeded expectations:

- 250 units sold in first 4 months!
- Estimated 800 units/year
- Projected 5 year supply will sell out in FY11

Objective study of method biases

Harmonization of measurement results

Measurement traceability

Candidate reference measurement procedure*

(Courtesy of Karen Phinney, NIST)

* Manuscript being submitted to Analytical Chemistry
Use of a common standard improves the performance of liquid chromatography-tandem mass spectrometry methods for serum 25-hydroxyvitamin-D

Graham D Carter and Julia C Jones, Clinical Chemistry Department, Imperial College Healthcare NHS Trust, Charing Cross Hospital, London W6 8RF, UK

Background: Liquid chromatography-tandem mass spectrometry (LC-MS/MS) is becoming increasingly popular for measuring 25-hydroxyvitamin-D (25-OH-D). Results submitted to the International Quality Assessment Scheme (DEQAS) have shown poor interlaboratory agreement. We investigated whether the use of a common standard would reduce interlaboratory imprecision.

Methods: A commercial standard and two controls were distributed with the DEQAS samples in January 2008. Participants were asked to calculate the results of samples and controls using their usual standard and the commercial standard. A method questionnaire was also distributed.

Results: Use of a common standard reduced the mean interlaboratory imprecision (coefficient of variation [CV]) for total 25-OH-D from 16.4% (in-house standards) to 10.4% (common standard). For 25-OH-D$_3$ and 25-OH-D$_2$, the mean CVs were reduced from 16.7% and 21.1% to 8.5% and 12.6%, respectively. Mean values obtained for total 25-OH-D using the common standard were higher by 6.1%.

Conclusions: Use of a common standard improved agreement among laboratories using LC-MS/MS methods for 25-OH-D. This suggests that problems with assay standardization contribute to interlaboratory imprecision. This may be related to the nature of the matrix used for working standards or errors in the calibration of stock standard solutions of 25-OH-D. Some participants used a gravimetric method, others UV spectrophotometry, to establish the concentration of stock solutions. Among the latter group there was uncertainty over the molar absorption coefficient of 25-OH-D solutions. We conclude that LC-MS/MS is not yet sufficiently robust to become the reference method for 25-OH-D and that gas chromatography-mass spectrometry might be a more suitable candidate.
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Are 25-OH-D$_2$ and 25-OH-D$_3$ bio-equivalent?

- Most *in vitro* findings suggest 25-OH-D$_2$ & 25-OH-D$_3$ and their active forms are biologically equivalent.
- Data supporting bioequivalence of D$_2$/D$_3$ at curing rickets.
- Recent results suggest that smaller doses (1000-1500 IU/d) are bio-equivalent at raising 25-OH-D levels.
- Vitamin D$_2$ is less toxic than vitamin D$_3$.
- Some evidence that 50,000 IU doses of vitamin D$_2$ are less effective than vitamin D$_3$ for raising 25-OH-D level.
FIG. 2. Time course of the rise in serum 25OHD after a single oral dose of 50,000 IU of either cholecalciferol (vitamin D3) or ergocalciferol (vitamin D2) to two groups of 10 normal men each.
CYP24A1

A. Human intestinal microsomes

B. CYP3A4

Jones et al
Vitamin D Workshop
Brugge, Oct 2009

products of 1α,25-(OH)₂D₂

products of 1α,25-(OH)₂D₃

CYP24A1 products of 1α,25-(OH)₂D

products of 25-OH-D₃

products 25-OH-D₂
ARE VITAMIN D₂ AND D₃ EQUIPOTENT?

- BOTH CURE RICKETS EQUALLY WELL
- AT PHYSIOLOGICAL LEVELS METABOLISED AT A SIMILAR RATE
- AT PHARMACOLOGICAL LEVELS D₂ COMPOUNDS METABOLISED FASTER PROBABLY AS THE RESULT OF CYP3A4 ACTION
- MAY EXPLAIN THE OBSERVED LOWER TOXICITY OF VITAMIN D₂
SO WHAT SHOULD WE MEASURE?

Total 25-OH-D or separate [25-OH-D$_2$] & [25-OH-D$_3$]?

- Total 25-OH-D is the clinically-important parameter
- Total 25-OH-D will generally suffice to assess health
- Plasma 25-OH-D$_2$ may be useful as a marker of dietary D or to assess the effectiveness of supplemental vitamin D$_2$ (the only source of prescription vitamin D in US)
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COST AND FREQUENCY OF 25-OH-D TESTING?

- COST OF 25-OH-D TESTING IS A HEALTH CARE-BURDEN
  - 25-OH-D TESTING & REPLETION MAY SAVE $$$$$

- WHAT IS THE IDEAL FREQUENCY OF 25-OH-D TESTING?
  - $t_{1/2}$ IS 15-20 DAYS

- GIVEN CURRENT D REPLETION TOOLS:
  - TEST AT BASELINE AND ABOUT 4 MONTHS
  - IF REPLETION HAS OCCURRED EVERY 6 MONTHS

- CAN ALL TESTING BE AVOIDED?
  - POOR RESPONDERS eg high BMI
FACTORS AFFECTING VITAMIN D INTAKE

Skin Synthesis

Clothing Coverage
Sunscreen Use
Season
Tanning Bed Use
Latitude
Sun Vacation
Time Spent Outdoors
Skin Pigmentation
Age

Dietary Intake

Fortified Foods
Supplement Use
Natural Sources

Vitamin D → 25-OH-D

BMI
Fat Malabsorption
Age
Summary

• Emergence of extra-renal 1α-hydroxylase emphasizes the value of serum 25-OH-D assay as a tool to monitor vitamin D status

• Performance of 25-OH-D assays has gradually improved but still has a long way to go. Introduction of NIST standards may improve.

• Research suggests that vitamin D₂ and D₃ have different rates of metabolism especially at pharmacological concentrations

• Repletion of 25-OH-D levels complicated by factors such as BMI, age and GI problems making monitoring of 25-OH-D important