Vitamin D: How good are our assays?

Ronda Greaves
Overview

- Background
- Clinical
- Reference intervals
- Measurement systems
- Approaches to quality
The Vitamin Alphabet

- A - Retinol
- B - group of 8
- C - Ascorbic acid
- D - Ergocalciferol
  - Cholecalciferol
- E - Tocopherol
- K - Phylloquinone

Total: 13 = 4 fat soluble + 9 water soluble

( ) indicates B group compound no longer classified as vitamins
Vitamin D: Definitions

**VITAMIN**

An organic compound required as a nutrient, which cannot be synthesized in adequate amounts, and therefore must be obtained in the diet

**HORMONE**

A chemical secreted by a group of cells (gland) into the circulation to affect the function of cells, through interaction with their receptors, in another part of the body
Vitamin D:

- **Vit D1** - is a 1:1 mixture of lumisterol and vitamin D2.

- **Vit D2 – ERGOCALCIFEROL**
  - Plant origin
  - Arises from ultraviolet irradiation of ergosterol
  - Cleaved at the 9,10 bond & develops a double bond b/w C-10 & 19

- **Vit D3 – CHOLECALCIFEROL**
  - Animal origin
  - Formed by breakage of the 9,10 bond in 7-dehydrocholesterol by ultraviolet irradiation, yielding a double bond b/w C-10 and C-19
  - Found in the skin, fur, and feathers of animals and birds exposed to sunlight, and also in butter, brain, fish oils, and egg yolk
Vitamin D

7-dehydrocholesterol

25-Hydroxylase
Liver

25OHD

1α-Hydroxylase
Kidney

1,25(OH)₂D³

24-Hydroxylase

24,25(OH)₂D³

1,24,25(OH)₃D₃
Clinical Utility

- Classically
  - Rickets
  - Osteomalacia

- Modern era
  - Bone health
  - Diabetes
  - Autoimmune diseases
  - Immune regulation
  - Infections
  - Cancer
  - Cardiovascular disease

Photos from Lehninger, Principles of Biochemistry
Increasing testing numbers

“In 2009, US laboratories were reporting surges in the number of vitamin D tests being ordered - increases of 50% to even 100%. But beyond the growth in testing and usage, what's the quality required by this type of testing?”

www.Westgard.com
Vitamin D: What level is appropriate?

- RCH
  - 1990’s: Reference range quoted 23 to 90 nmol/L
  - 2000’s: Change to recommended range of 50 to 150 nmol/L

- Other ranges
  - >60 nmol/L proposed based on rise in PTH
  - >75 nmol/L proposed for health
  - >100 nmol/L for cancer prevention

- On going debate of what range is needed for health

- **BUT** – we don’t have harmonisation of methods!!!!
Pre analytical factors

- Biological Variation
- Seasonal variation
- Skin pigmentation
- Racial differences

- Vitamin D is stable in whole blood stored at room temperature in sunlight for up to 96 hours.

(Poster: AACB ASM in 2005)
Vitamin D

- Choice for routine assessment of vitamin D status
- Need to extract to remove Vit D from DBP (Vit D binding protein)
- Standards calibrated against D3
- Some immunoassays cross react with D2
  - Traditionally considered an advantage
- Supplementation in Australia originally D2 now mainly D3

Vitamin D₂ Is Much Less Effective than Vitamin D₃ in Humans

Laura A. G. Armas, Bruce W. Hollis, and Robert P. Heaney
Creighton University (L.A.G.A., R.P.H.), Omaha, Nebraska 68131; and Medical University of South Carolina (R.W.H.), Charleston, South Carolina 29425
Vitamin D: Automated analysis

- **Roche Cobas e601**
  - 25 OH Vit D3 only
  - 0% cross reactivity with 25 OH Vit D2

- **Diasorin Liaison**
  - 25 OH Vit D3
  - >80% cross reactivity with 25 OH Vit D2
Vitamin D: Other Immunoassays

- Enzyme Linked Immunosorbent Assay
- Radio-immunoassay
  - Diasorin (Sorin)
  - IDS

NEW AUTOMATED
- IDS – ISYS platform
- Abbott – recent lab trials
- Siemens – under development

Chromatography + MS (+MS)

- Gold standard
- TAT a problem
- Expertise required
- Up front cost high
INTRODUCTION

Several recent studies have shown that vitamin D deficiency is common in adults and children around the world. In addition to the well-known effects of vitamin D deficiency, such as calcium malabsorption, there is growing evidence that the risk for other conditions (e.g. cancers) may be increased. The measurement of 25-
hydroxyvitamin D (25(OH)D) is accepted as the clinical indicator of vitamin D status and is important in the diagnosis and treatment of vitamin D deficiency. The major issue with immunoassays is that they cannot differentiate between the two forms of 25(OH)D: 25(OH)D2 & 25(OH)D3, and instead, rely on the cross-reactivity of the antibody to measure a total 25(OH)D concentration. If that cross-reactivity is less than 100% then vitamin D2 therapy may not be monitored effectively. The aim of this study was to develop a quantitative method for 25(OH)D2 and 25(OH)D3 in serum to prevent the misdiagnosis of vitamin D deficiency in patients.

METHODS

A Waters® ACQUITY® Tandem Quadrupole Detector (TQD) coupled to an ACQUITY UPLC® (Waters Corporation, Manchester, UK) was used for all analyses (Figure 1). The 25(OH)D compounds were separated from endogenous interferences using an ACQUITY UPLC HILIC C18 Column 2.1 x 50 mm, 1.7 µm employing a gradient elution profile, 73.99% in 1.5 minutes following a 2 minute initial hold at a flow rate of 0.45ml/min, where mobile phase A and B are 2M ammonium acetate+0.1%formic acid in water and methanol respectively.

The instrument was operated in positive electrospray ionisation mode using Masslynx™ 4.1 software with auto data processing by the Quanlynx™ Application Manager. Specific Multiple Reaction Monitoring (MRM) experiments for each compound were carried out as shown in Table 1.

A single calibrator and bi-level QC's (Chromsystems, Munich, Germany) were prepared as per the manufacturer's instructions. A low QC was prepared by pooling human serum and adding a known concentration of 25(OH)D2 and 25(OH)D3. The final concentrations of the low, medium and high QC samples were 19, 27 and 54ng/ml for 25(OH)D2 and 13, 26 and 89ng/ml for 25(OH)D3 respectively.

RESULTS

Accuracy

The accuracy of the assay was determined by the analysis of external quality control samples from DEQAS (www.deqas.org). The Chromsystems single point calibrator was used and a calibration line constructed through zero to calculate the DEQAS sample concentrations. Passing-Bablok linear regression was used to compare the Waters 25(OH)D3 results with the DEQAS LC/MS method mean. All results were within ±1.1% deviation of the expected value (Figure 2).

DISCUSSION

A method for the UPLC/MS/MS analysis of 25(OH)D2 and 25(OH)D3 in serum has been developed. The methodology involves a simple liquid-liquid extraction of the analytes from serum and the HRE detection of each analyte using two transitions. Quantifier and qualifier ion ratios were monitored to ensure lack of interferences. The assay demonstrates good sensitivity with acceptable intra and inter-day precision. Using this methodology it is feasible to manually process and analyse up to 100 samples per day.

CONCLUSION

- A method for the independent quantification of 25(OH)D2 and 25(OH)D3 in serum has been developed with good linearity, sensitivity and precision.
- UPLC/MS/MS offers significant advantages over the traditional HPLC/UV methodology through reduced sample volume, increased sensitivity, specificity and speed.
- UPLC/MS/MS allows for the accurate and reliable measurement of 25(OH)D2 and 25(OH)D3 in serum to prevent the misdiagnosis of vitamin D deficiency in patients who are receiving vitamin D2 supplementation.

References

Method comparisons
2009 QAP end of cycle 32 report

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Your Method Code: I: Electrochemiluminescence 11L Roche Diagnostics E170i e 601 (cobas 6000-IA) 21: Roche Diagnostics (Hitachi) C: LC-MS-MS
Jan 2010 DEQAS: Vitamin D

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LC-MS/MS Reference methods

• Various LC-MS/MS methods available
• Variation between these methods
• Roche assay based on the method below
• This was developed in cooperation with Dr. Vogeser Klinikum Grosshadern
• Then further optimized at Roche

Information provided courtesy of Roche Australia
Vitamin D3 chromatography + MS/MS

Vitamin D3 (25-OH) elutes at approx. 8 min.

Total gradient time: 20 min. incl. extensive column cleaning

Information provided courtesy of Roche Australia
Current challenges in vitamin D standardization

- There is considerable variability in reference methods. Lack of a “real” vitamin D standard reference material which can be used for immunoassays.

- Variability in methods for reference standardization (methodological risks, influence of chromatographic resolution).

- No “real” reference values existing.
# NIST human serum SRM

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Vit D Released 2009
SRM 972

Allows for a common primary calibrator
Current challenges in vitamin D standardization

- **Limitations of NIST controls** SRM 972:
  - Level 1: native human serum
  - Level 2: level 1 diluted with horse serum
  - Level 3: human serum spiked with vitamin D2 (25-OH)
  - Level 4: human serum spiked with vitamin D3 (25-OH) and 3-epi 25(OH)

- **In vitro anomaly affecting immunoassays**
  - Exogeneously added vitamin D does not distribute to the vitamin D binding protein (VDBP) as it occurs as in vivo
  - Exogeneously added material binds to other moieties than the VDBP

- **failure of quantitative recovery in immunoassays**

- **Is there a way out of this dilemma?**

*Information provided courtesy of Roche Australia*
NIST SRM 972 standard is detectable by the Roche LC-MS/MS

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Information provided courtesy of Roche Australia
How safe are LC-MS/MS data? The Quest Story

08. Jan 2009 - New York Times:

- “Quest acknowledges errors in vitamin D tests”

- The nation's largest medical laboratory company provided possibly erroneous results to thousands of people who had their vitamin D levels tested in the last two years, the company has acknowledged.

- Quest's problems with the vitamin D analysis arose after it shifted in 2006 and 2007 to a new test of its own design, replacing an older F.D.A.-approved test.

- The new test promised to be more accurate and offer more detailed information, Quest executives said. But the test relied on a sophisticated instrument called a mass spectrometer, which can be tricky to use, especially for high-volume testing.

Information provided courtesy of Roche Australia
Specifications for trueness and precision of a reference measurement system for serum/plasma 25-hydroxy vitamin D analysis

Clinica Chimica Acta, 2009; 408: 8-13
Dietmar Stöckl, Patrick M. Sluss and Linda M. Thienpont

Abstract

Background

The divergence in analytical quality of serum/plasma 25-hydroxy-vitamin D analysis calls for defining specifications for a reference measurement system.

Methods

Fundamentally, in a reference measurement system, there should be a relationship between the analytical specifications for higher- (reference) and lower-order (routine) measurements. Therefore, when setting specifications, we started with limits for routine imprecision ($CV_{rou}$) and bias ($B_{rou}$) using 4 models: (1) the misclassifications in diagnosis, (2) biological variation data (reference interval (RI) and monitoring), (3) expert recommendations, and (4) state-of-the-art performance. Then, we used the derived goals to tailor those for reference measurements and certified reference materials (CRMs) for calibration by setting the limits for $CV_{ref}$ at 0.5 $CV_{rou}$, $B_{ref}$ at 0.33 $B_{rou}$, max. uncertainty ($U_{max}$) at 0.33 $B_{ref}$

Results

The established specifications ranged between $CV_{rou} \leq 22\%$, $B_{rou} \leq 10\%$, $CV_{ref} \leq 11\%$, $B_{ref} \leq 3.3\%$, $U_{max} 1.1\%$ (model 3) and $CV_{rou} \leq 4\%$, $B_{rou} \leq 2.6\%$, $CV_{ref} \leq 2\%$, $B_{ref} \leq 0.9\%$, $U_{max} 0.3\%$ (model 2, monitoring).

Conclusions

Model 2 (monitoring) gave the most stringent goals, model 3, the most liberal ones. Accounting for state-of-the-art performance and certification capabilities, we used model 2 (RI) to recommend achievable goals: for routine testing, $CV_{rou} \leq 10\%$, $B_{rou} \leq 5\%$, for reference measurements, $CV_{ref} \leq 5\%$, $B_{ref} \leq 1.7\%$, and for CRMs, $U_{max} 0.6\%$.

Keywords: Serum/plasma 25-hydroxyvitamin D2; Serum/plasma 25-hydroxyvitamin D3; Quality goals; Bias; Imprecision; Uncertainty
Stockl et al: Approaches for quality

- Four different approaches
- Followed the 1999 Stockholm consensus conference guidelines on quality specifications

1. Clinical Interpretation: Analyses the impact of bias and analytical imprecision on interpretation of results based on clinical decision limits
2. Biological Variation: Relates analytical performance to the intra- and inter-individual biological variation of vitamin D
3. Expert Opinion: Considered performance goals set by expert opinion from external quality assurance programs
4. State of the art: Evaluated the literature on currently used measurement procedures for vitamin D analysis with stated recovery and imprecision data.
Stockl et al: What they found

- Routine measurement systems Expert opinion gave the most liberal goals i.e. 5x the CV’s for the biological variation goals

- Expert opinion CV 22% Bias 10%

- Biological Variation CV 4% Bias 2.6%

- Survey of 14 "state of the art“ studies of methods
  - only one method was close to achieving the performance required by the Biological Variation model i.e. the most stringent model.
Stockl *et al*: Recommendations

- Using the biologic variation approach i.e. Gowan model

- Routine Testing
  - CV ≤ 10%
  - Bias ≤ 5%

- Reference Measurements
  - CV ≤ 5%
  - Bias ≤ 1.7%

- Westgard: “The requirements are challenging, but Stock *et al* believe the laboratory community is up to the challenge”
Moves for Harmonisation

- LC-MS/MS for standardization of Vitamin D assays needs
  - high analytical investment
  - critical interpretation of data (especially in HPLC method validation)
  - accurate and reliable methods

- LC-MS/MS methods for standardization can only be compared if specific mass transitions are used
Summary

- Vitamin D: How good are our assays?
  - Clinical understanding of the importance of vitamin D has increased in recent years
  - Both automated immunoassays and chromatography MS methods have developed
  - Discussion surrounds acceptable performance of vitamin D assays
  - Harmonisation is the next objective