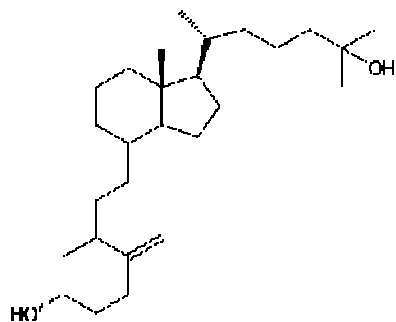


25-Hydroxyvitamin D Assay Analytical Issues



Rosemary L Schleicher, PhD

Nutritional Biomarkers Branch

Division of Laboratory Sciences

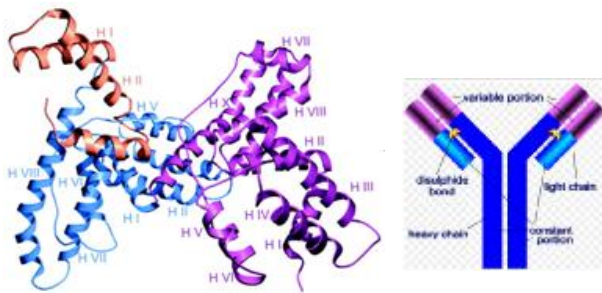
National Center for Environmental Health

Presentation to DRI Working Group

March 26, 2009



Types of Analytical Methods for Measuring 25-Hydroxyvitamin D



Protein Binding Assays

- Vitamin D Binding Protein
- Antibodies



Chemical Assays

- HPLC-UV
- GC-MS
- LC-MS/MS

Types of Analytical Methods for Measuring 25-Hydroxyvitamin D



Protein Binding Assays

- RIA (DiaSorin, IDS)
- EIA (IDS)
- CLIA (DiaSorin)
- ECL (Roche)
- CPBA (Nichols)



Chemical Assays

- HPLC-UV
- GC-MS
- LC-MS/MS

Analytical Method Performance Specifications

Scientific

- Accuracy
- Precision
- Specificity
- Sensitivity
- Ruggedness

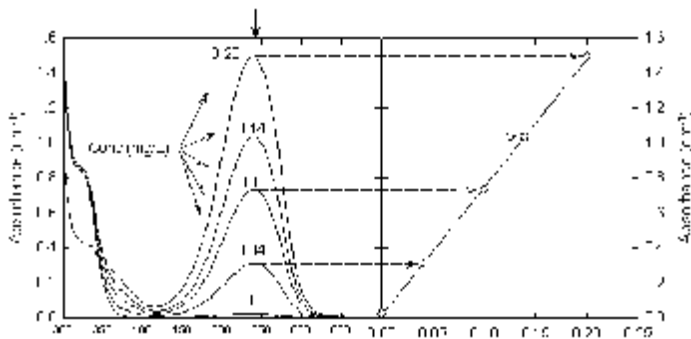
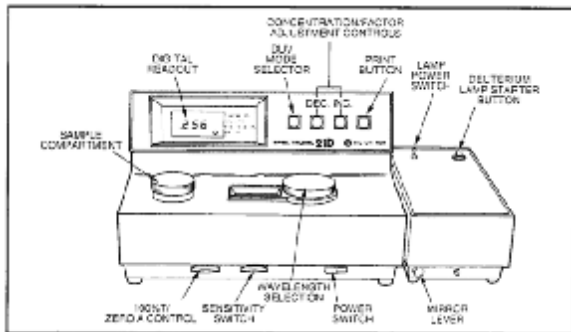


Practical

- Cost
- Throughput
- Sample Volume (μL)
- Multi-analyte Capability
- Safety
- Staff Skills



Accuracy



- Purified powdered 25OHD2 and 25OHD3 are dissolved in organic solvent and the concentration is calculated based on the absorption at peak wavelength
- Standard solutions are generally mixed with protein to prepare calibrators
- All assays use this general approach

Accuracy and clinical implications of seven 25OHD methods compared with LC-MS/MS as a reference

HJ Roth et al Ann Clin Biochem 45:153, 2008

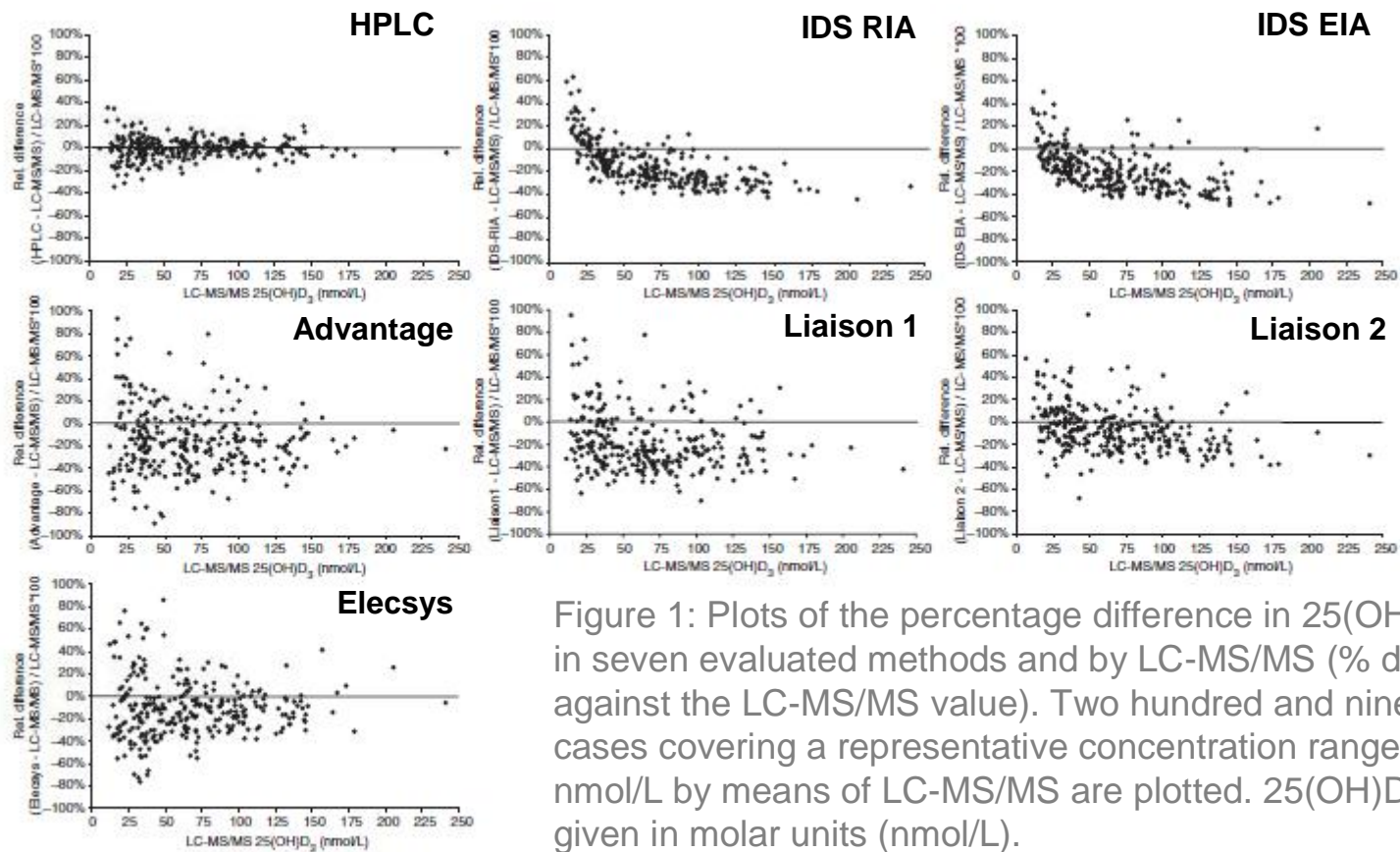
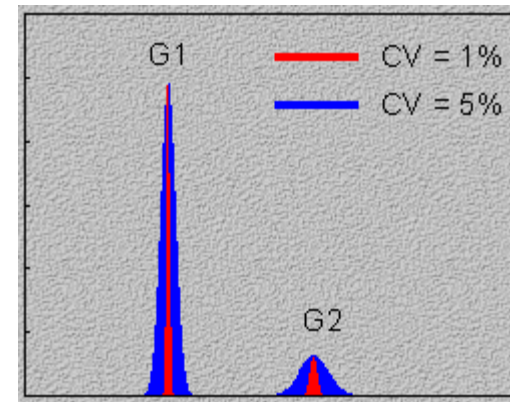
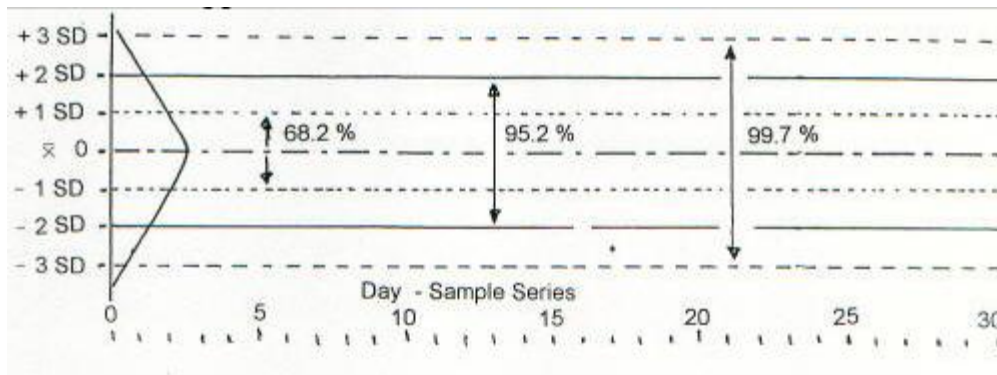


Figure 1: Plots of the percentage difference in 25(OH)D₃ levels measured in seven evaluated methods and by LC-MS/MS (% differences are plotted against the LC-MS/MS value). Two hundred and ninety-one complete cases covering a representative concentration range between 6.5 and 240 nmol/L by means of LC-MS/MS are plotted. 25(OH)D₃ concentrations are given in molar units (nmol/L).

Precision

- QC pools (L, M, H) are tested in every assay
- Concentrations spanning expected range of measurements
- Limits are developed over minimum of 20 days (mean \pm SD)
- Coefficient of variation (CV) normalizes the SD to the mean ($SD/Mean*100$)



Precision

- Analytical precision (CV_A) needed for an assay has been related to variation within individuals (CV_I)
- CV_I estimate for 25OHD = 11.3% (NHANES 2000-2002, D Lacher, personal communication)
- Quality specification goals that are recommended
 - Optimal $CV_A = 0.25 CV_I$ \implies 2.8%
 - Desirable $CV_A = 0.50 CV_I$ \implies 5.7%
 - Minimal $CV_A = 0.75 CV_I$ \implies 8.5%

CDC Analytical Coefficients of Variation (CV_A) for 25OHD Percentage (mean in ng/mL)

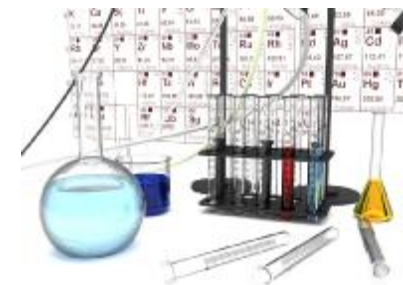
	RIA	LC-MS/MS [§]	
	25OHD	25OHD3	25OHD2
Low QC	12.6% (10)	7.5% (12)	
Medium QC	9.7% (23)	6.4% (21)	10.7% (3)
High QC	11.8% (40)	7.9% (35)	9.1% (10)

Optimal: 0.25 CVI = 2.8% Desirable: 0.50 CVI = 5.7% Minimal: 0.75 CVI = 8.5%

§revised method

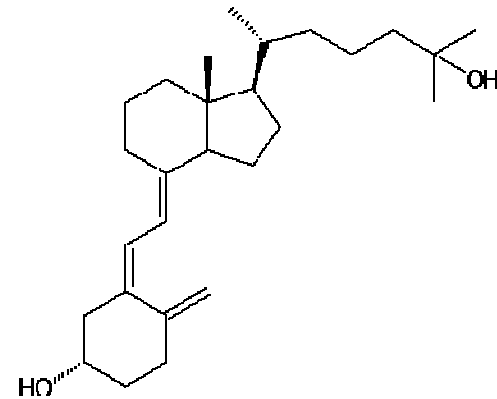
Specificity

- **Protein binding assays**
 - Selective extraction from matrix
 - Competition of substances likely to interfere
 - Spike potential interferences
- **Chemistry-based assays**
 - Selective extraction from matrix
 - Different types of chromatography (LC, GC) to separate compounds of interest from other compounds (retention time window for peak of interest)
 - Different types of detection (UV, MS, MS/MS) to specifically measure the compounds of interest
 - Increasing specificity from UV to MS to MS/MS
 - Examine peak shapes
 - Spike potential interferences



Sensitivity

- Limit of detection (LOD) is the concentration at which 25OHD3 and/or 25OHD2 have a 95% probability of being >0 (S/N=3)
 - 25OHD3, all results >LOD
 - 25OHD2, many results <LOD
- Protein binding assays:
 - 25OHD, 0.8-4 ng/mL
- Chemistry-based assays:
 - 25OHD3, 0.5-2 ng/mL
 - 25OHD2, 0.5-2 ng/mL



Proficiency Testing

- **DEQAS** – 4 exercises per year x 5 specimens per exercise

- 478 participating labs - Jan, 2009
- DiaSorin Liaison 172
- IDS EIA 140
- LC-MS 44
- DiaSorin RIA 42

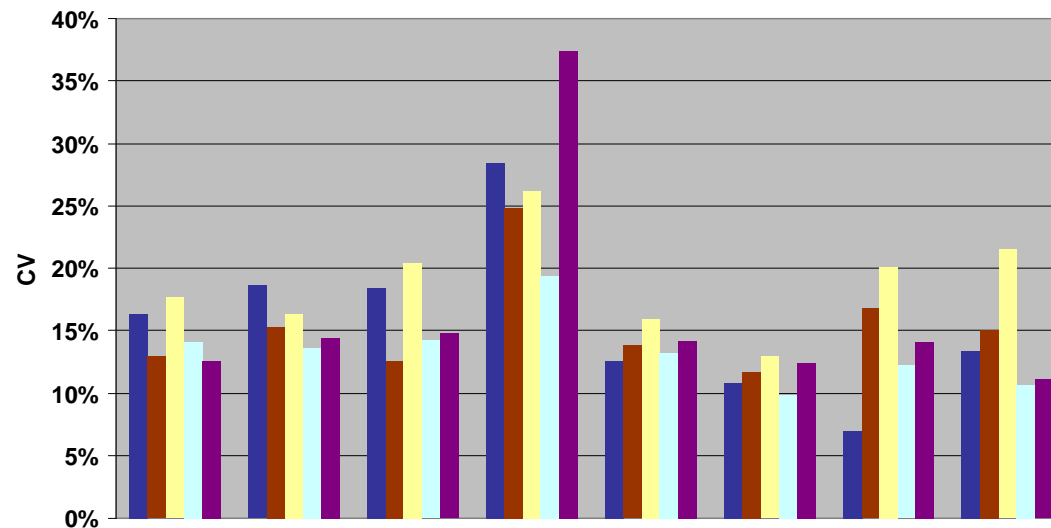
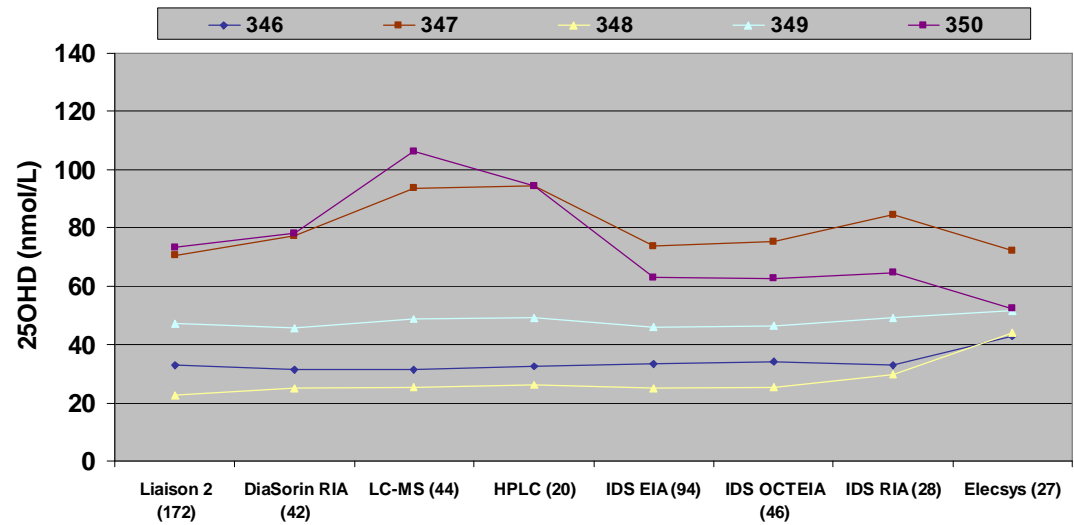


- **CAP** – 2 exercises per year x 2 specimens per exercise

- 107 participating labs - Oct, 2008
- DiaSorin Liaison 59
- DiaSorin RIA 19
- LC-MS 14
- IDS EIA 9



January 2009 DEQAS Exercise



NHANES

Why has CDC switched from
DiaSorin RIA to LC-MS/MS?



DiaSorin RIA Analytical Issues

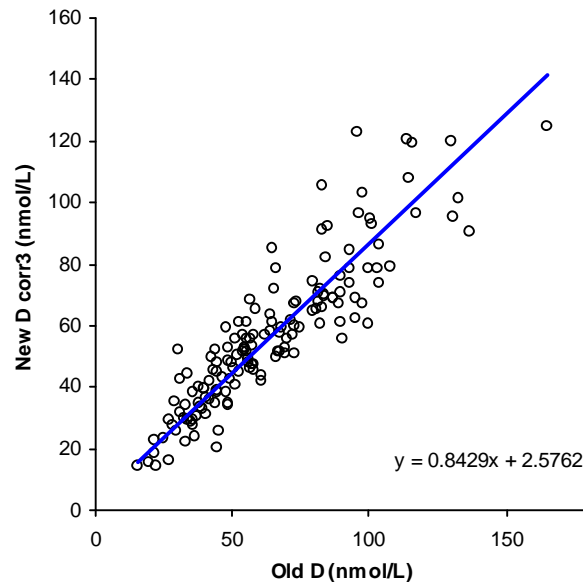
Based on CDC experience with the DiaSorin RIA assay, there are two issues with negative implications with regards to NHANES or other long-term studies:

1. Reformulation of the DiaSorin RIA kit in the late 1990
2. Wide acceptable QC limits “permitting” QC pool mean shifts over time



1. Reformulation of the DiaSorin RIA Kit

- New antibody with improved binding was introduced in the late 1990s
- Old Diasorin RIA kit was used for NHANES III (1988-1994)
- New Diasorin RIA kit was used for NHANES 1999-2006
- In 2004, the CDC laboratory reanalyzed a subset of 150 banked serum samples (stored at -70°C) from NHANES III with the new version of the RIA over a 3-mo period to assess assay differences



Deming Regression: Data obtained with the new RIA were on average 11.7% lower than data obtained with the old RIA (after adjusting for temporal assay shift)

2. QC Pool Shifts Over Time

- Manufacturer QC pool limits are wide
- CDC lab has generated and characterized in-house QC pools to allow judgment of runs based on stricter limits

QC Pool	Mean (ng/mL)	2SD Limits (ng/mL)	CV (%)
Diasorin Low	15	9.0 - 21	20
Diasorin High	58	35 - 81	20
QC Pool	Mean (ng/mL)	2SD Limits (ng/mL)	CV (%)
CDC Low	9.5	7.3 - 12	12
CDC Medium	23	19 - 28	9
CDC High	40	32 - 48	10

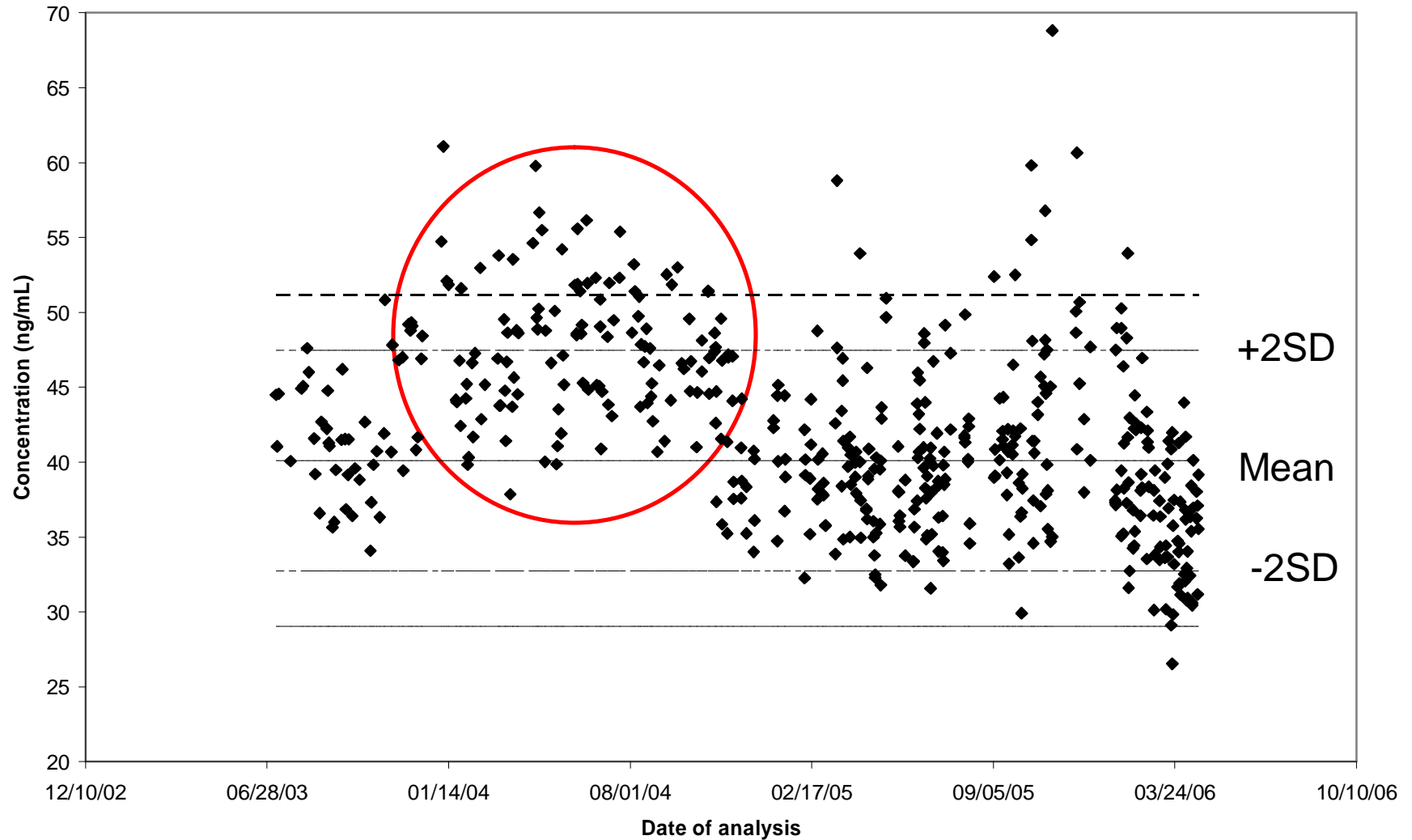
2. QC Pool Shifts Over Time

- We observed small shifts in assay performance due to changes in reagent and calibrator lots over a period of years
- Most noticeable shift occurred during 2004 and necessitated readjustment of our in-house QC limits

QC Pool	Mean (ng/mL)	2SD Limits (ng/mL)	CV (%)	N	Limits used
CDC Low	13	9.3 - 17	14.1%	24	7/8/03-1/28/04
CDC Medium	20	15 - 24	11.3%		
CDC High	40	33 - 48	9.2%		
CDC Low	13	10 - 17	12.8%	77	1/31/04-3/30/04
CDC Medium	20	16 - 24	9.4%		
CDC High	42	34 - 50	9.9%		
CDC Low	14	9.9 - 17	13.5%	107	4/1/04-4/19/06
CDC Medium	21	16 - 25	10.5%		
CDC High	43	34 - 53	11.0%		

2. QC Pool Shifts Over Time

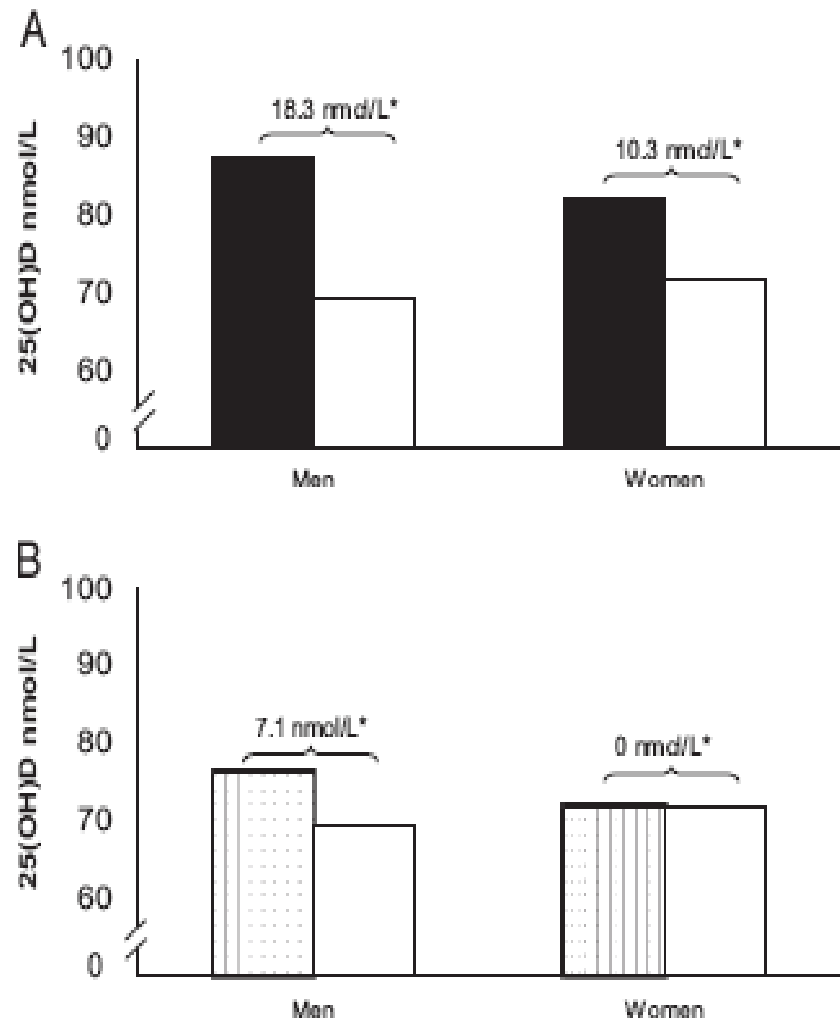
Vitamin D - Bench QC - NH0303 High - Plotted against a limits



Effects on NHANES Interpretation

- Interpretation of NHANES time trends was difficult because of these effects
- After taking all necessary assay adjustments into consideration, the difference in 25OHD values between NHANES III and NHANES 2003-2004 was 10-11 nmol/L

Looker et al., Am J Clin Nutr 2008



Circulating 25-Hydroxyvitamin D Levels and Survival in Patients With Colorectal Cancer

Kimmie Ng, Jeffrey A. Meyerhardt, Kana Wu, Diane Feskanich, Bruce W. Hollis, Edward L. Giovannucci, and Charles S. Fuchs

J CLIN ONCOL 2008; 26(18): 2984

“Although all samples were assayed at the same laboratory, cases from the 1992, 1994, and 1996 NHS questionnaires were assayed in 2000, those from the 1998 and 2000 questionnaires in 2003, and all HPFS samples in 2005. Plasma 25(OH)D levels were lower in the 2000 assay compared with the 2003 assay; among 12 quality control samples from the same plasma pool, mean 25(OH)D values were 19.2 ng/mL in 2000 and 22.8 ng/mL in 2003.¹⁴ Therefore, **quartile cutoffs for statistical analyses were determined separately for each laboratory run.** Furthermore, an indicator variable for laboratory run was included in analyses that used plasma 25(OH)D as a continuous variable.”

How will new NHANES 25OHD data be compared to the past? (2001-2006) versus (2007 and beyond)

NHANES DiaSorin RIA Results

2001	100 samples (25 per 2001 quartile) assayed over 1 year (evenly distributed)
2002	100 samples (25 per 2002 quartile) assayed over 1 year (evenly distributed)
2003	100 samples (25 per 2003 quartile) assayed over 1 year (evenly distributed)
2004	100 samples (25 per 2004 quartile) assayed over 1 year (evenly distributed)
2005	100 samples (25 per 2005 quartile) assayed over 1 year (evenly distributed)
2006	100 samples (25 per 2006 quartile) assayed over 1 year (evenly distributed)

LC-MS/MS Testing Plan

Test 10 samples per year = 60 samples/assay * 10 assays

Each equation based on 10 LC-MS/MS assays

Deming Regression

e.g., [LC-MS/MS] = $m \times [2001 \text{ RIA}] + b$



Accuracy-Based Improvement for 25OHD Assays



- Four NIST standard reference materials are expected to be commercially available by summer 2009
 - SRM 972 (serum-based) – 4 levels
 - SRM 2972 (solvent-based) – 1 level each for 25OHD2 and 25OHD3

SRM 972 LC-MS/MS Values

nmol/L	NIST	NIST	NIST	CDC*	CDC/NIST
	25OHD3	3-epi-25OHD3	Total 25OHD3	25OHD3	
Level 1	60.1	3.5	63.6	64.0	1.01
Level 2	31.2	1.9	33.1	33.7	1.02
Level 3	46.6	2.7	49.3	50.9	1.03
Level 4	83.7	98.9	182.6	190.1	1.04

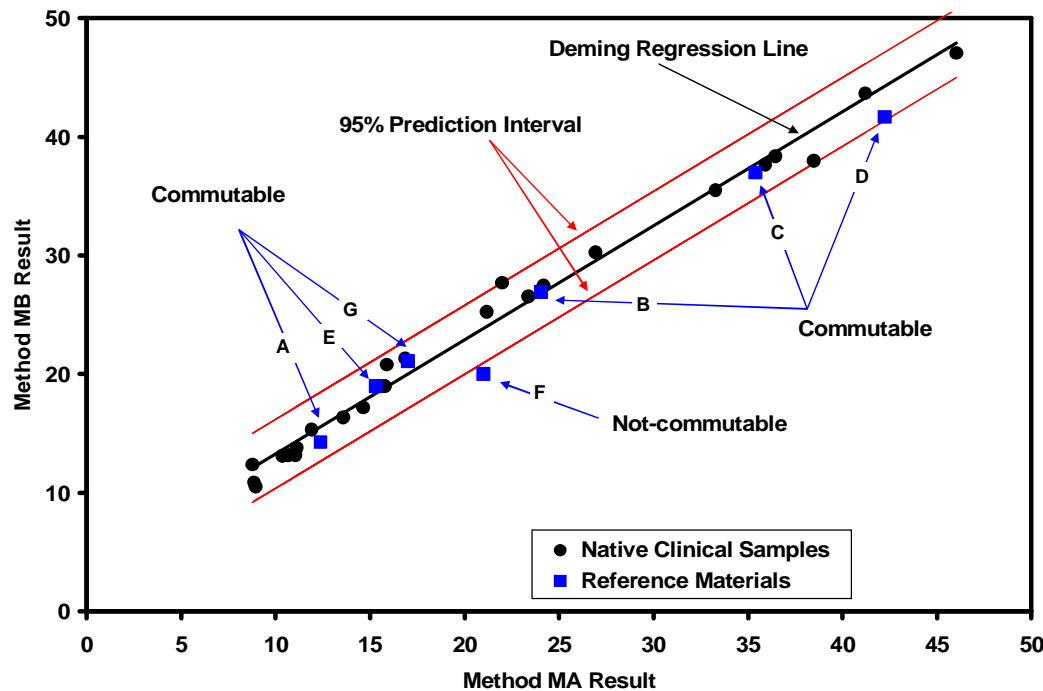
*CDC method does not differentiate 3-epimer

SRM 972 LC-MS/MS Values

nmol/L	NIST 25OHD2	CDC 25OHD2	CDC/NIST
Level 1	1.3	1.2	0.92
Level 2	4.2	5.4	1.29
Level 3	65.0	68.6	1.06
Level 4	5.7	6.3	1.11



Commutability Issue



Relationship between lower order immunoassay and higher order LC-MS/MS is the same for reference materials as it is for unaltered patient specimens at similar concentrations



Characterization and Qualification of Commutable Reference Materials for Laboratory Medicine; Proposed Guideline C53-P

Acknowledgements

Christine M Pfeiffer, CDC
Leslie F McCoy, CDC
Huiping Chen, CDC
Donna LaVoie, CDC
Madhu Chaudhary-Webb, CDC
Karen W Phinney, NIST
David A Lacher, CDC

For more information

Email: RSchleicher@cdc.gov

Web: www.cdc.gov/NCEH/DLS/

Nutrition Report: <http://www.cdc.gov/nutritionreport/>

