Analytical Variability Among Methods for the Measurement of 25-Hydroxyvitamin D

Still Adding to the Noise

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ABSTRACT

Objectives: *To compare total 25-hydroxyvitamin D [25(OH)D] results measured by 3 direct immunoassays, including the previous version of the DiaSorin Liaison2 assay and the current versions of the Siemens Centaur2 and the Abbott Architect assays, with results measured in serum extracts by liquid chromatography/tandem mass spectrometry (LC/MS) and radioimmunoassay (RIA).*

Methods: *Our study sample consisted of 163 consecutive clinical specimens submitted to our laboratory for 25(OH)D testing.*

Results: *Regression and bias analyses of the data revealed that results measured by the 3 direct immunoassay methods had high degrees of random variability and bias relative to the results determined by LC/MS and RIA. The relative biases between results measured by the direct assays and the comparison methods exceeded a recommended criterion for the total allowable error of a 25(OH)D test in as many as 48% of our clinical specimens. Of the subjects in our study sample, 33, 37, 30, 45, and 71 were classified as vitamin D deficient based on results determined by LC/MS, RIA, Liaison2, Architect, and Centaur2, respectively.*

Conclusions: *Intermethod variability in 25(OH)D assays continues to limit our progress toward the establishment of reference values for 25(OH)D in health and our efforts to gain a better understanding of the role of vitamin D insufficiency as a risk factor for disease.*

A marked increase in laboratory testing for 25-hydroxyvitamin D [25(OH)D] has been fueled by an increased focus on the diagnosis and treatment of osteoporosis,[1] the demonstration of a high prevalence of vitamin D deficiency in many populations,[2] and the discovery that the biological significance of vitamin D extends far beyond its classic roles in the regulation of bone and mineral metabolism.[3] The increased demand for 25(OH)D testing has led to the introduction of new methods based primarily on direct nonisotopic competitive protein binding (CPB) and high-performance liquid chromatography (HPLC) with tandem mass spectrometry detection (LC/MS). As the number of available methods increases, it is especially important to ensure that the degree of standardization and harmonization of the different methods is sufficient to provide high-quality test results that will facilitate the development of a strong evidence base that can be used to develop robust reference values for the evaluation of vitamin D insufficiency and to more precisely define the role of vitamin D as a risk factor for disease.

The evolution of CPB methods began with the description of a manual assay that used a rat vitamin D–binding protein and tritiated 25(OH)D_3 to measure total 25(OH)D [t25(OH)D] in ethanol extracts of human serum, culminating with the development of various automated chemiluminescent immunoassays for the analysis of unextracted specimens.[4-8] During this same period, methods that used HPLC with UV detection to separately quantitate 25(OH)D_2 and 25(OH)D_3 in serum extracts[9] were further enhanced by the use of mass fragmentography for detection,[10] the implementation of increasingly more sophisticated chromatographic and MS techniques, and the automation of sample preparation and data analysis.[11] The establishment of standard reference materials for 25(OH)D

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and the elevation of 2 candidate LC/MS methods to the status of reference method procedures (RMPs)[12,13] are expected to play important roles in improving the quality of all currently available 25(OH)D assays.[14] The need for increased accuracy is especially apparent in the case of the direct immunoassay methods, as attested to by the steady stream of primary publications and editorials[15-18] that have described the limitations of this particular method of analysis.

In November 2010, we discovered that the DiaSorin Liaison 25(OH)D method (Liaison2; DiaSorin, Stillwater, MN) was affected by heterophile antibody interference that caused falsely elevated test results in approximately 20% of our patients.[19] Because this defect (which was soon confirmed by another laboratory[20]) was neither formally disclosed nor addressed by the manufacturer, we transferred our testing to a reference laboratory that measured 25(OH)D by LC/MS. We carried out a method comparison study between the LC/MS and the Liaison2 to evaluate the effect of the method change on the continuity of patient results and later had occasion to evaluate the direct immunoassays developed for the Siemens Centaur XP (Centaur2, Siemens Healthcare Diagnostics, Tarrytown, NY) and the Abbott Architect i2000 (Abbott Laboratories, Abbott Park, IL). This report compares t25(OH)D results determined by 3 direct immunoassay methods with results determined by LC/MS and by the DiaSorin radioimmunoassay (RIA). Preliminary accounts of portions of this work have been presented in poster format at 2 national meetings.[21,22]

Materials and Methods

Clinical Specimens

Method comparison studies were carried out using 163 consecutive serum specimens (123 female, 40 male) submitted to the laboratory for the measurement of t25(OH)D on March 2, 2011, and March 9, 2011. The sera that remained after the requested clinical testing had been performed were stored frozen at –30°C prior to testing by immunoassay. During the data analysis phase of our investigation, the patient samples were divided into 2 subgroups based on their 25(OH)D_2 concentrations as determined by LC/MS. The specimens in 1 group (D_3) had 25(OH)D_2 concentrations that were less than 4.0 ng/mL (the lower limit of detection of the LC/MS method) and thus contained primarily 25(OH)D_3. The specimens in the second group (D_2D_3) contained both 25(OH)D_2 and 25(OH)D_3. Two specimens were excluded from the groups because their results for 25(OH)D_2 and 25(OH)D_3 had not been recorded. Some demographic characteristics of the patients in our study and the 25(OH)D concentrations in the 2 subgroups of specimens are summarized in **Table 1**. This study was carried out in accord with the ethical standards established by the institutional review board of the Loyola University Health Sciences Division.

Methods for the Measurement of 25(OH)D

DiaSorin Liaison2

This direct chemiluminescent immunoassay for t25(OH)D was performed with the second-generation reagent formulation used by clinical laboratories in the United States between 2007 and January 20, 2012, and by clinical laboratories outside the United States between 2007 and September 10, 2011, at which times the reagents in the 2 markets were modified to eliminate false-positive results owing to heterophile antibody interference. This method is reported to have relative cross-reactivities with 25(OH)D_3, 25(OH)D_2, and 3-epi-25(OH)D_3 of 100%, 104%, and less than 1%, respectively; an analytical measuring range of 4 to 150 ng/mL; and run-to-run coefficients of variation (CVs) in the range of 6% to 13%.[23]

Table 1
25-Hydroxyvitamin D [25(OH)D] Concentrations in the Study Sample and in Groups D_3 and D_2D_3

	All Samples (n = 163[a])			Group D_3 [25(OH)D_2 < 4.0 ng/mL] (n = 94[b])			Group D_2D_3 [25(OH)D_2 ≥ 4.0 ng/mL] (n = 67[c])		
	Median	Minimum	Maximum	Median	Minimum	Maximum	Median	Minimum	Maximum
Age, y	55	8	90	52	12	88	61	8	90
LC/MS									
Total 25(OH)D, ng/mL	31	8	74	25	8	53	39	11	74
25(OH)D_3, ng/mL	22	<4	53	25	8	53	13	<4	53
25(OH)D_2, ng/mL	<4	<4	68	<4	<4	<4	23	4	68
D_2/D_3 ratio	1.5	<0.1	15.5						
Total 25(OH)D, ng/mL									
RIA	26	6	60	24	7	50	31	6	60
Liaison2	33	6	67	29	6	58	35	7	67
Centaur2	23	6	94	18	6	68	33	6	94
Architect	24	7	58	24	9	56	24	7	58

LC/MS, liquid chromatography/mass spectrometry; RIA, radioimmunoassay.
[a] Includes 123 females and 40 males.
[b] Includes 67 females and 27 males.
[c] Includes 54 females and 13 males.

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Siemens Centaur XP

The Centaur2 direct chemiluminescent assay for t25(OH)D was performed using the Centaur XP immunoassay system. This method was approved by the Food and Drug Administration (FDA) on October 19, 2011. It is reported to have cross-reactivities with 25(OH)D_3, 25(OH)D_2, and 3-epi-25(OH)D_3 of 100.7%, 104.5%, and 1.1%, respectively; an analytical measuring range of 3.7 to 150 ng/mL; and run-to-run CVs in the range of 4.8% to 11.1%.[24] The Centaur2 results were obtained prior to August 2012, when the manufacturer assigned new values to the calibrators to increase the values of all results by 4.1 ng/mL.[25]

Abbott Architect i2000

This direct chemiluminescent microparticle immunoassay for the measurement of t25(OH)D was FDA approved on November 30, 2011. The method is reported to have cross-reactivities with 25(OH)D_3, 25(OH)D_2, and 3-epi-25(OH)D_3 of 105%, 82%, and 2.7%, respectively; an analytical measuring range of 13 to 96 ng/mL; and run-to-run CVs of less than 5%. According to the manufacturer, a sample with a t25(OH)D result of more than 96 ng/mL must be reported as such and cannot be diluted and reanalyzed.[26]

Total 25(OH)D assays by the 3 direct immunoassays were performed in our institution's clinical laboratory, where each method was operated exactly as described in the manufacturer's directional insert. Prior to any public presentation of our data, we gave each manufacturer a copy of the results that showed how its own immunoassay performed relative to the LC/MS method.

LC/MS

The 25-hydroxyvitamin D by LC/MS (vitamin D, 25Hydroxy, LC/MS/MS: test 17306) was performed by Quest Diagnostics (Wood Dale, IL). The reportable results for this test included the concentrations of 25(OH)D_2, 25(OH)D_3, and t25(OH)D [calculated as the sum of 25(OH)D_2 and 25(OH)D_3]. The method, which is used at all of Quest's regional laboratories, has been shown to provide results that are in close agreement with the National Institute of Standards and Technology (NIST) target values for 25(OH)D_2 and 25(OH)D_3 for Standard Reference Material 972, levels I to III.[27] The analytical measuring ranges for both 25(OH)D_2 and 25(OH)D_3 are 4 to 512 ng/mL. The total imprecision for 25(OH)D_2 and 25(OH)D_3 measurements at the regional laboratory was reported to be less than 7% at concentrations in the range of 14 to 90 ng/mL.

RIA

t25(OH)D was determined using the ^{125}I RIA Kit (DiaSorin). In this method, 25(OH)D_2 and 25(OH)D_3 are extracted from serum with acetonitrile, and the t25(OH)D concentration of the extract is determined using an equilibrium RIA. Since its commercialization in 1993, this assay has served as an "unofficial" reference method and has set the standard for the clinical diagnosis of vitamin D deficiency.[15] It has been shown to correlate well with many highly regarded methods, including HPLC-UV[28] and many different LC/MS methods.[29-31] This RIA is reported to have 100% cross-reactivity with both 25(OH)D_3 and 25(OH)D_2, a run-to-run CV of less than 11% at t25(OH)D concentrations in the range of 9 to 49 ng/mL, and an analytical measuring range of 2.5 to 100 ng/mL.[32] This method does not cross-react with 3-epi-25(OH)D_3.[29] The RIA assays were performed by the manufacturer at its analytical laboratory in Stillwater, MN.

Method Comparison Study

The correlations between t25(OH)D results determined by the 2 comparison methods, LC/MS and RIA (x variables), and results determined by each of the 3 direct immunoassay methods (y variables) were evaluated by linear regression analysis. Random variability between the test and comparison methods was evaluated by inspection of the x,y plots compared with the lines of identity and by calculation of the coefficient of determination (r^2) and the standard error of the regression (SE). The presence of proportional or constant systematic error was declared when the 95% confidence intervals of the slope or intercept of a regression line excluded the value of 1.0 or 0.0, respectively. Paired *t* tests were used to evaluate the significance of the mean intermethod biases for each comparison. The ranges of the intermethod biases observed for individual patient specimens served as additional measures of random intermethod variability.

Bias plots of the percent differences [(t25(OH)$D_{\text{Test Method}}$ – t25(OH)$D_{\text{Comp Method}}$)/t25(OH)$D_{\text{Comp Method}}$] × 100 for each specimen were used to separately evaluate intermethod differences in analytical variability for D_3 and D_2D_3 specimens. These plots were also used to determine the proportion of patient results for which the intermethod bias between a test and a comparison method exceeded an established criterion (±25%) for the total allowable error of a 25(OH)D test.[33]

Recovery of 25(OH)D_2

The effect of the 25(OH)D_2 concentration of a sample on the intermethod bias in t25(OH)D measured by the 3 direct immunoassays was evaluated by plotting the percent bias (calculated as described above) relative to each comparison method against the 25(OH)D_2 concentration that was measured by LC/MS for the group of 67 D_2D_3 specimens. The relationships between the 2 variables were modeled using locally weighted scatter plot smoothing with a tension of 0.85.

Concordance of Clinical Interpretations of Test Results

The concordance between the direct immunoassays and the comparison methods for the classification of a patient's

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vitamin D status as deficient (<10 ng/mL), insufficient (10-29 ng/mL), or sufficient (≥30 ng/mL) relative to a set of widely accepted clinical decision levels,[34] as well as relative to the Institute of Medicine (IOM) guidelines[35] of deficient (<12 ng/mL) , insufficient (12-19 ng/mL), and sufficient (>20 ng/mL), was evaluated using Cohen's κ statistic. κ values greater than 0.80 indicate near complete; more than 0.60, strong; more than 0.4, moderate; and more than 0.20, fair agreement beyond chance.[36] All statistical analyses described above were performed using Systat 11 (Systat Software, Chicago, IL).

Results

Initial Evaluation of Intermethod Variability

The scatter plots of t25(OH)D results determined by the direct immunoassays and the comparison methods revealed high degrees of intermethod variability as indicated by the substantial deviations of individual data points from the lines of identity **Figure 1**. In addition, statistically significant random and systematic errors, statistically significant positive or negative mean biases, and broad ranges in the relative biases for individual patient specimens were observed when the direct IAs were compared with LC/MS and RIA **Table 2**. For example, Liaison2 demonstrated a large amount of random error (r^2 = 0.62; SE, 8.5 ng/mL) as well as proportional (slope, 0.79) and constant errors (intercept, 7.7 ng/mL) relative to LC/MS. Liaison2 also demonstrated a high degree of random error and significant proportional error compared with RIA. The preponderance of positively biased outliers among Liaison2 results in samples with LC/MS or RIA results greater than 20 ng/mL (Figures 1A and 1B) is evidence of the heterophile antibody interference that was previously shown to affect this method.[19]

The Centaur2 results (Figures 1C and 1D) also showed extreme outliers relative to those obtained by both comparison methods, especially in samples with t25(OH)D results greater than 50 ng/mL. Centaur2 results showed obvious nonlinear relationships with those determined by both comparison methods. The nonlinear relationship with LC/MS was confirmed in an independent sample of 95 randomly selected clinical specimens that were analyzed using a different lot of Centaur reagents (data not shown). Therefore, the regression and subsequent bias analyses were performed separately for subgroups of samples with 25(OH)D values less than 30 ng/mL and 30 ng/mL or more. The SE of the regression of Centaur2 on LC/MS for samples with results less than 30 ng/mL was 6.7 ng/mL. No proportional or constant bias was observed for samples in this subgroup, but the mean bias was –5 ng/mL (minimum, –15.4 ng/mL; maximum, 41.0 ng/mL). A large SE was also observed for samples in the higher range, as well as proportional and constant errors and a mean bias of –4.1 ng/mL (minimum, –24.0 ng/mL; maximum,

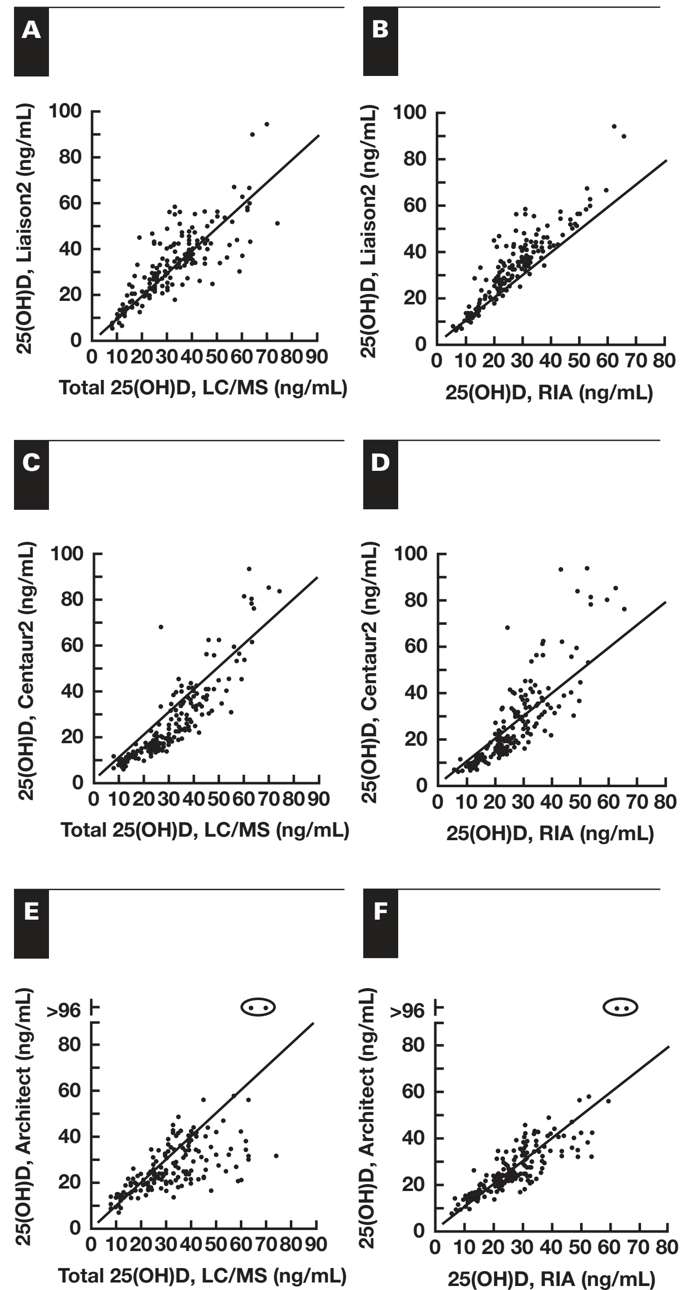

Figure 1 Relationships between total 25-hydroxyvitamin D [25(OH)D] concentrations measured by direct immunoassay, liquid chromatography/mass spectrometry (LC/MS), and radioimmunoassay (RIA). Results measured by Liaison2 (DiaSorin, Stillwater, MN) (**A**, **B**), Centaur2 (Siemens Healthcare Diagnostics, Tarrytown, NY) (**C**, **D**), or Architect (Abbott Laboratories, Abbott Park, IL) (**E**, **F**) for 163 clinical specimens were plotted against the results measured by LC/MS or RIA. The line of identity (y = x) is displayed on the scatter plots of the data. The regression statistics for each comparison are presented in Table 2. The circled data points in panels **E** and **F** exceeded the analytical measuring range for the Architect (96 ng/mL) and could not be repeated after dilution per the manufacturer's instructions.

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Table 2
Random and Systematic Errors and Bias in Direct Immunoassays for Total 25-Hydroxyvitamin D [t25(OH)D][a]

	Linear Regression				Bias (y – x)		
Comparison Method (x)/Samples	n	r^2 (SE)	Slope (95% CI)	Intercept (95% CI), ng/mL	Mean (95% CI), ng/mL	Minimum, ng/mL	Maximum, ng/mL
Liaison2 (y)							
LC/MS (all)	163	0.62 (8.5)	0.79[b] (0.69 to 0.89)	7.7[b] (4.3 to 11.1)	0.9 (–0.5 to 2.3)	–28.8	25.7
RIA (all)	162	0.80 (6.1)	1.12[b] (1.04 to 1.20)	2.4 (–0.2 to 4.9)	5.7[c] (4.7 to 6.7)	–4.4	28.1
Centaur2 (y)							
LC/MS							
All	163	0.76 (8.7)	1.12[b] (1.02 to 1.22)	–8.3[b] (–11.7 to –4.8)	–4.5[c] (–5.8 to –3.1)	–24.0	41.0
<30 ng/mL	71	0.34 (6.7)	0.76 (0.50 to 1.02)	–0.3 (–5.5 to 5.0)	–5.0[c] (–6.6 to –3.4)	–15.4	41.0
≥30 ng/mL	92	0.69 (9.4)	1.40[b] (1.20 to 1.60)	–20.5[b] (–28.9 to –12.0)	–4.1[c] (–6.2 to –1.9)	–24.0	31.7
RIA							
All	162	0.67 (10.3)	1.33[b] (1.18 to 1.48)	–8.4[b] (–12.8 to –4.1)	0.4 (–1.3 to 2.1)	–17.5	49.9
<30 ng/mL	99	0.49 (7.2)	1.16 (0.92 to 1.40)	–4.9 (–9.9 to 0.1)	–1.7[b] (–3.1 to –0.3)	–10.5	43.5
≥30 ng/mL	63	0.43 (13.8)	1.58[b] (1.12 to 2.04)	–18.2[b] (–36.0 to –0.4)	3.7[b] (0.1 to 7.3)	–17.5	49.9
Architect (y)							
LC/MS (all)	163	0.41 (7.8)	0.47[b] (0.39 to 0.55)	11.4[b] (8.4 to 14.5)	5.4[c] (–7.0 to –3.7)	–42.1	13.8
RIA (all)	162	0.69 (5.6)	0.77[b] (0.69 to 0.85)	5.7[b] (3.4 to 8.1)	–0.5 (–1.5 to 0.4)	–22.0	14.4
RIA (y)							
LC/MS (all)	162	0.79 (5.0)	0.70[b] (0.65 to 0.76)	4.6[b] (2.6 to 6.6)	–4.9[c] (–5.9 to –3.9)	–27.1	6.5

CI, confidence interval; LC/MS, liquid chromatography/mass spectrometry; RIA, radioimmunoassay.
[a] Data from the split-sample analysis of the study samples by the 3 immunoassays and the comparison methods were analyzed by linear regression and the paired *t* test as described in the Materials and Methods section. Because of the obvious nonlinear relationships between the Centaur2 method and both of the comparison methods, correlation and bias analysis were performed separately on subgroups of specimens with t25(OH)D concentrations less than 30 ng/mL and 30 ng/mL or more.
[b] The slope or intercept of a regression line differs significantly from the value of 1.0 or 0.0, respectively.
[c] The mean bias calculated by the paired *t* test was significantly different from 0.0 ng/mL, $P < .01$ (2-tailed).

31.7 ng/mL). The SEs of the regression of Centaur2 on RIA showed high degrees of random error in both the low and high subgroups. No constant or proportional errors were observed for the low subgroup, but both types of systematic error were observed for the high subgroup. The mean overall biases relative to RIA were –1.7 ng/mL (minimum, –10.5 ng/mL; maximum, 43.5 ng/mL) and 3.7 ng/mL (minimum, –17.5 ng/mL; maximum, 49.9 ng/mL) in the low and high subgroups, respectively.

The regression of the Architect on LC/MS showed an extreme amount of random error ($r^2 = 0.41$; SE, 7.8 ng/mL), both proportional and constant errors, and a mean bias of –5.4 ng/mL (minimum, –42.1 ng/mL; maximum, 13.8 ng/mL). The comparison between Architect and RIA also revealed the presence of random error, proportional and constant errors, and a mean intermethod bias of –0.5 ng/mL (minimum, –22.0 ng/mL; maximum, 14.4 ng/mL). Two specimens that had results of more than 96 ng/mL by the Architect assay (see circled data points in Figures 1E and 1F) had results of approximately 70 ng/mL when measured by either LC/MS or RIA, suggesting that the Architect results may have been falsely increased by interfering substances in the sample matrices.

Effect of 25(OH)D_2 Concentration on Intermethod Variability

The results of our initial evaluation indicated that there were especially high levels of analytical variability in specimens that had t25(OH)D results greater than 40 ng/mL. Since values in this portion of the analytical range are more likely to be observed in patients who have received pharmacological doses of vitamin D_2, we separately performed bias and regression analyses on the D_3 and D_2D_3 specimen groups. Bias plots of the results measured by Liaison2 and LC/MS showed that the D_2D_3 specimens were more likely to have large percent negative biases relative to LC/MS than D_3 specimens **Figure 2A**. Such a relationship was not observed in the Liaison2/RIA comparison **Figure 2B**. The bias plots for Centaur2 showed variable but largely negative percent biases for D_3 specimens relative to both comparison methods. For D_2D_3 specimens, the magnitudes of the negative biases decreased and eventually turned positive as the t25(OH)D concentration of specimens increased **Figure 2C** and **Figure 2D**. These trends suggested that the Centaur2 assay preferentially reacted with 25(OH)D_2 when both 25(OH)D_2 and 25(OH)D_3 were present in a specimen. The bias plots comparing results measured by Architect and the 2 comparison methods showed many instances of extreme percent positive biases in D_3 specimens **Figure 2E** and **Figure 2F** for samples in the lower portion of the analytical measuring range, suggesting the presence of a standardization/calibration defect or a common, positive interfering substance that was not measured by either LC/MS or RIA. The 25(OH)D_2-related increases in the incidence and magnitude of negative percent biases relative to both comparison methods in D_2D_3 specimens are

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not surprising since the manufacturer's claims state that the method demonstrates a cross-reactivity for 25(OH)D_2 of only 80%.[26] The dashed lines in the bias plots denote one of the more conservative of the recommended thresholds (±25%) for the total allowable error (TEa) of a 25(OH)D method.[33] We found that the biases between results determined by Liaison2, Centaur2, and Architect and results determined by LC/MS exceeded the recommended TEa in 31%, 48%, and 40%, respectively, of the clinical specimens submitted to our laboratory for 25(OH)D testing. The intermethod biases between results determined by the 3 direct immunoassays and results determined by RIA exceeded the thresholds in 30%, 45%, and 26%, respectively, of our specimens.

Separate analysis of D_3 and D_2D_3 specimens by linear regression and the paired *t* test revealed significant intergroup differences in the types and magnitudes of analytical variability relative to the comparison methods **Table 3**. The Liaison2 method showed a positive bias relative to LC/MS for group D_3, whereas there was a negative bias and a proportional error for group D_2D_3. The RIA and LC/MS comparisons demonstrated negative mean biases and proportional errors for both groups of specimens. The Centaur2 and RIA comparisons revealed a negative bias for D_3 specimens and both a positive bias and a positive proportional error for the D_2D_3 specimens. The Architect and LC/MS comparison showed a marked increase in random variability in D_2D_3 specimens compared with D_3 specimens. This difference was accompanied by an increase in negative proportional error and a large negative average bias relative to that observed for D_3 specimens. The Architect and RIA comparison for D_2D_3 specimens also showed an increase in negative proportional error and a significant negative mean bias compared with those observed for D_3. These data suggested that the direct immunoassays tended to under- or overrecover 25(OH)D_2 relative to the comparison methods.

The RIA and LC/MS comparisons for D_3 specimens showed a relatively low degree of random error, a negative proportional error, and a mean bias of –1.5 ng/mL (minimum, –8.5 ng/mL; maximum, 6.5 ng/mL). In contrast, comparisons for the D_2D_3 specimens showed an increased intermethod random variability, an increase in the magnitude of the negative proportional error, and a negative mean bias of –9.5 ng/mL (minimum, 27.1 ng/mL; maximum, 6.5 ng/mL). It is not clear whether this finding indicates an underrecovery of 25(OH)D_2 by RIA, an overestimation of t25(OH)D by LC/MS, or some combination of the two.

Recoveries of 25(OH)D_2 by Immunoassay

Further evaluation of the D_2D_3 specimens by plotting the percent bias between the t25(OH)D results determined by the direct IAs and the comparison methods against the 25(OH)D_2 concentration of the specimen **Figure 3** showed that the

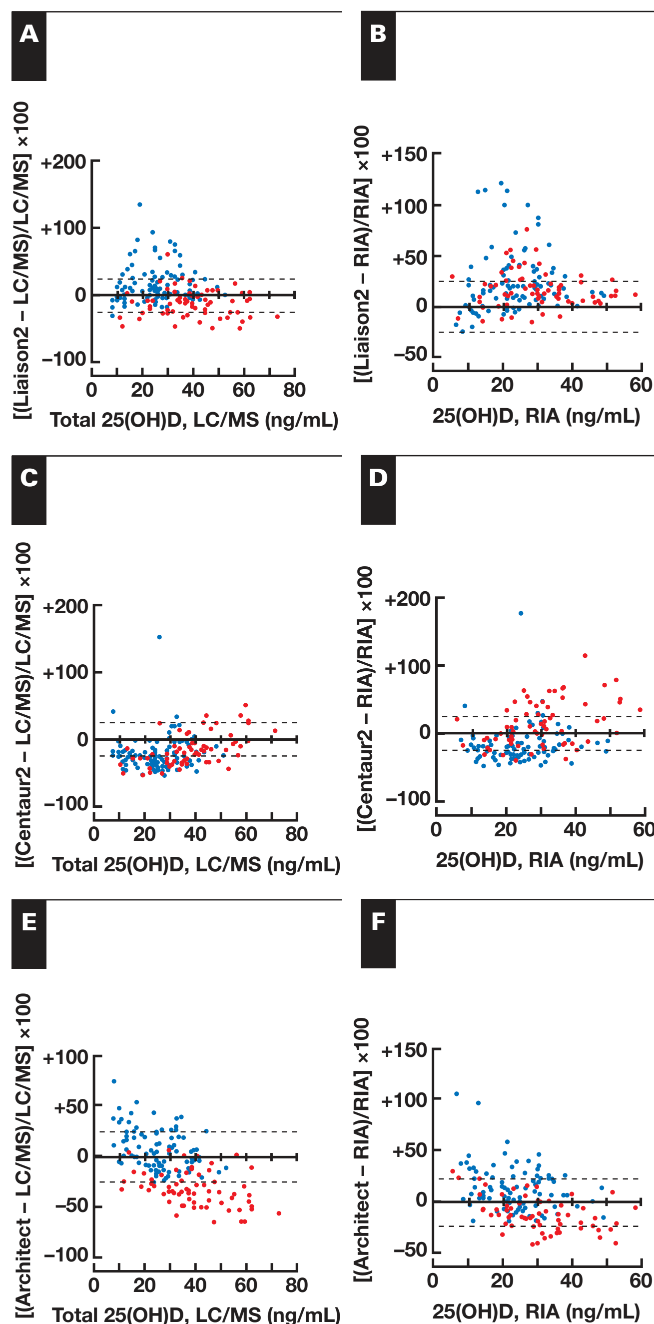

Figure 2 Intermethod biases between 3 direct immunoassays and liquid chromatography/mass spectrometry (LC/MS) and radioimmunoassay (RIA) comparison methods. Percent biases between the Liaison2 (DiaSorin, Stillwater, MN) (**A**, **B**), Centaur2 (Siemens Healthcare Diagnostics, Tarrytown, NY) (**C**, **D**), and Architect (Abbott Laboratories, Abbott Park, IL) (**E**, **F**) and the LC/MS and RIA comparison methods are displayed for a group (D_3) of samples that contained primarily 25-hydroxyvitamin D_3 [25(OH)D_3; blue dots] and a group (D_2D_3) that contained both 25(OH)D_2 and 25(OH)D_3 (red dots). The dotted parallel lines in each graph mark the upper and lower limits of the total allowable error (±25%) for 25(OH)D results that will be interpreted relative to population-based reference limits.

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Table 3
Random and Systematic Error and Bias Compared With LC/MS and RIA for Specimens Containing Primarily $25(OH)D_3$ (Group D_3) and Specimens Containing $25(OH)D_2$ and $25(OH)D_3$ (Group D_2D_3)[a]

Test Method (y)	r^2 (SE)	Slope (95% CI)	Intercept (95% CI), ng/mL	Mean (95% CI), ng/mL	Minimum, ng/mL	Maximum, ng/mL
LC/MS (x), group D_3 (n = 94)						
Liaison2	0.73 (7.1)	1.12 (0.98 to 1.26)	1.6 (–2.3 to 5.6)	4.7[c] (3.2 to 6.2)	–5.0	25.7
Centaur2	0.61 (7.1)	0.87 (0.73 to 1.01)	–1.7 (–5.6 to 2.3)	–5.1[c] (–6.6 to –3.6)	–20.0	41.0
Architect	0.79 (4.8)	0.91 (0.81 to 1.01)	3.4[b] (0.7 to 6.1)	1.0 (–0.1 to 2.0)	–8.9	13.8
RIA	0.92 (2.7)	0.89[b] (0.83 to 0.94)	1.4 (–0.1 to 2.9)	–1.5[c] (–2.1 to –0.9)	–8.5	6.5
LC/MS (x), group D_2D_3 (n = 67)						
Liaison2	0.65 (8.3)	0.80[b] (0.66 to 0.94)	3.6 (–2.6 to 9.8)	–4.4[c] (–6.5 to –2.3)	–28.8	18.7
Centaur2	0.78 (9.9)	1.33[b] (1.15 to 1.51)	–16.7[b] (–24.2 to –9.3)	–3.7[b] (–6.3 to –1.0)	–24.0	31.7
Architect	0.45 (7.4)	0.47[b] (0.33 to 0.61)	7.1[b] (1.6 to 12.7)	–14.1[c] (–16.7 to –11.6)	–42.1	2.0
RIA	0.73 (6.2)	0.72[b] (0.61 to 0.83)	1.6 (–3.1 to 6.3)	–9.5[c] (–11.3 to –7.7)	–27.1	3.3
RIA (x), group D_3 (n = 93)						
Liaison2	0.71 (6.8)	1.21[b] (1.05 to 1.37)	1.0 (–2.8 to 4.8)	6.2[c] (4.7 to 7.6)	–2.3	28.1
Centaur2	0.60 (7.1)	0.92 (0.76 to 1.08)	–1.8 (–5.8 to 2.3)	–3.6[c] (–5.1 to –2.2)	–17.5	43.5
Architect	0.81 (4.5)	0.99 (0.89 to 1.09)	2.8[b] (0.2 to 5.4)	2.5[c] (1.5 to 3.4)	–7.9	14.4
RIA (x), group D_2D_3 (n = 67)						
Liaison2	0.88 (4.8)	1.10 (1.00 to 1.20)	2.1 (–1.2 to 5.4)	5.1[c] (3.8 to 6.3)	–4.4	21.0
Centaur2	0.70 (11.6)	1.48[b] (1.24 to 1.72)	–9.0[b] (–16.8 to –1.1)	5.8[c] (2.7 to 8.9)	–17.3	49.9
Architect	0.77 (4.8)	0.73[b] (0.63 to 0.83)	3.7[b] (0.5 to 6.9)	–4.7[c] (–6.0 to –3.3)	–22.0	4.9

CI, confidence interval; LC/MS, liquid chromatography/mass spectrometry; RIA, radioimmunoassay; 25(OH)D, 25-hydroxyvitamin D.
[a] Random and systematic errors between the test and comparison methods were reevaluated for specimens in groups D_3 ($25(OH)D_2 < 4.0$ ng/mL) and D_2D_3 ($25(OH)D_2 \geq 4.0$ ng/mL).
[b] The slope or intercept of a regression line differs significantly from the value of 1.0 or 0.0, respectively.
[c] The mean bias calculated by the paired *t* test was significantly different from 0.0 ng/mL, $P < .01$ (2-tailed).

positive bias of the Liaison2 relative to RIA, although highly variable, remained relatively constant as the $25(OH)D_2$ concentration of the sample increased. In contrast, the negative bias relative to LC/MS showed a tendency to increase with increasing $25(OH)D_2$. The Centaur2 demonstrated progressive increases in the positive bias relative to both the LC/MS and RIA methods as the $25(OH)D_2$ concentration of the specimen increased. The Architect showed progressive increases in the negative bias relative to both LC/MS and RIA as the $25(OH)D_2$ concentration of the specimen increased. These data

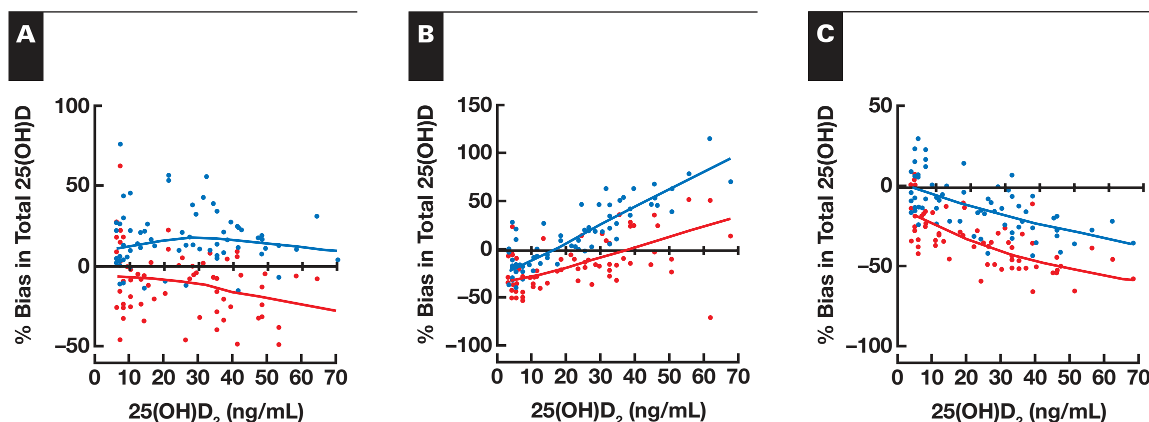

Figure 3 Relative biases between total 25-hydroxyvitamin D [t25(OH)D] results determined by direct immunoassays and liquid chromatography/mass spectrometry (LC/MS) or radioimmunoassay (RIA) as a function of the $25(OH)D_2$ concentration of the specimen for the Liaison2 (DiaSorin, Stillwater, MN) (**A**), Centaur2 (Siemens Healthcare Diagnostics, Tarrytown, NY) (**B**), and Architect (Abbott Laboratories, Abbott Park, IL) (**C**). The percent biases in t25(OH)D concentrations measured by a direct immunoassay relative to the concentrations determined by LC/MS (red dots) or RIA (blue dots) in the clinical specimens from group D_2D_3 were plotted as a function of the $25(OH)D_2$ concentration of specimen as determined by LC/MS. The relationship between each set of paired variables was modeled using locally weighted scatter plot smoothing with a tension of 0.85. The observation of a positive or a negative trend in a fitted line is consistent with an over- or an underrecovery, respectively, of $25(OH)D_2$ by a test method relative to LC/MS (red lines) or RIA (blue lines). Percent biases were calculated as described in the labels to the ordinates of the graphs in Figure 2.

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suggest that the Liaison2 method underrecovered 25(OH)D_2 relative to the LC/MS, while the Centaur2 and the Architect methods demonstrated significant, dose-dependent over- and underrecovery, respectively, of t25(OH)D in samples that contained 25(OH)D_2.

Clinical Concordance of t25(OH)D Results Determined by Different Methods

Clinical interpretations of vitamin D nutritional status based on t25(OH)D results determined by RIA and Liaison2 showed strong concordances with interpretations based on t25(OH)D results determined by LC/MS or RIA when interpretation was based on either consensus or IOM guidelines **Table 4**. In contrast, the concordances between interpretations based on Centaur2 and those determined by either LC/MS or RIA ranged from fair to moderate. The concordances between Architect results and LC/MS or RIA results ranged from moderate to strong.

Discussion

Our study showed that the 3 direct immunoassays for serum t25(OH)D had several important analytical limitations relative to 2 methods that measured t25(OH)D in extracted specimens. The most striking differences between results measured by the 2 categories of methods were the magnitudes and the wide ranges of the relative biases observed for individual clinical specimens. Interference by a heterophile antibody is typically a rare cause of inaccuracy that has the potential to affect any immunoassay.[37] Thus, it was surprising to discover that interference by this mechanism caused falsely elevated test results in as many as 20%[19] and 29%[20] of samples from patients tested by the Liaison2 method in Chicago, Illinois, and Liege, Belgium, respectively. In the present study, our comparison between Liaison2 and the comparison methods, both of which eliminated the possibility of heterophile antibody interference by the extraction/deproteinization of the specimen prior to analysis, showed that such interference is easily recognized by the presence of extreme outlying points in scatter plots of the paired observations. Thus, the presence of extreme outliers in a split-sample correlation study, however rare, should warrant additional studies to exclude the possibility of clinically significant heterophile antibody interference. While the Liaison2 assay is no longer available, an awareness of its analytical limitations is important because of its widespread use by laboratories that performed 25(OH)D testing between 2008 and January 2012.[38] A PubMed search between January 2008 and January 2012 identified 47 publications that cited the Liaison as a method of analysis, 21 of which were clinical studies investigating the relevance of 25(OH)D deficiency as a risk factor for disease. During this same period, the Liaison2 was used to analyze the specimens from national health and nutrition surveys performed in Canada, Germany, and the United Kingdom,[14] and it was used as the predicate method for the 510(k) submission to the FDA by the manufacturer of the Architect assay.[26] While the consequences of the inaccurate results that may have been reported by the Liaison2 have yet to be determined, there is no doubt that the noise caused by heterophile antibody

Table 4
Concordance of Clinical Interpretations of Vitamin D Nutritional Status Based on Results Determined By LC/MS, RIA, and Direct Immunoassay Methods[a]

	Clinical Interpretation (No. of Patients)			Agreement With Comparison Method, κ (SE)	
Method	**Deficient**	**Insufficient**	**Sufficient**	**LC/MS**	**RIA**
Consensus guidelines[b]					
LC/MS	33	47	83	—	0.695 (0.048)
RIA	37	63	62	0.695 (0.048)	—
Liaison2	30	41	92	0.664 (0.051)	0.626 (0.050)
Centaur2	71	32	60	0.440 (0.050)	0.467 (0.051)
Architect	45	62	56	0.460 (0.056)	0.587 (0.054)
IOM guidelines[c]					
LC/MS	9	28	129	—	0.725 (0.060)
RIA	13	24	125	0.725 (0.060)	—
Liaison2	11	19	133	0.682 (0.068)	0.699 (0.063)
Centaur2	28	43	92	0.339 (0.056)	0.411 (0.057)
Architect	6	39	118	0.570 (0.069)	0.649 (0.062)

IOM, Institute of Medicine; LC/MS, liquid chromatography/mass spectrometry; RIA, radioimmunoassay.
[a] Total 25-hydroxyvitamin D results measured by each of the 5 methods were interpreted according to clinical decision levels for vitamin D deficiency, insufficiency, and sufficiency as recommended by Consensus and Institute of Medicine guidelines. The strengths of the agreements between clinical classifications based on each immunoassay and each comparison method were evaluated using the κ statistic. The threshold above which a κ value signifies a near-complete, strong, moderate, or fair agreement between classifications based on 2 methods is 0.8, 0.6, 0.4, and 0.2, respectively. The dash indicates comparison method, κ not calculated.
[b] Deficient, <20 ng/mL; insufficient, 20-30 ng/mL; and sufficient, >30 ng/mL.
[c] Deficient, <12 ng/mL; insufficient, 12-19 ng/mL; and sufficient, ≥20 ng/mL.

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interference could have affected the results of the studies in which it was used as a measure of vitamin D nutrition.

We evaluated the Siemens Centaur XP and Abbott Architect i2000 methods with the intention of validating one or the other for use in our laboratory. However, both candidates demonstrated unacceptably high levels of random variability relative to both of our comparison methods. The presence of occasional extreme positively biased outliers suggested that both methods are affected by interfering antibodies or other proteins present in the sample matrix. An additional problem with the Centaur2 assay was its nonlinear dose-response relationship relative to results determined by either LC/MS or RIA. Other investigators[30,39] have demonstrated similar nonlinear relationships, indicating that a defect in standardization or calibration is a significant source of systematic error in the Centaur2 assay. This defect led to an overestimation of the prevalence of vitamin D deficiency in our patients. The manufacturer formally recognized the importance of this problem in August 2012 when it reassigned the assay's calibrator values to increase the reported results by an average of 4 ng/mL (95% confidence interval, –4 to 12).[25] While this change was apparently intended to "fix" (at least on average) the problem of false declarations of vitamin D deficiency or insufficiency relative to other 25(OH)D methods, it did not address the problem of random patient-to-patient variability in test results. However, it did add to the positive bias that was already present owing to the overrecovery of 25(OH)D_2.

The Architect assay had positive biases relative to both comparison methods at t25(OH)D concentrations less than 20 ng/mL and negative biases in samples with concentrations greater than 30 ng/mL. The biases in the low range appeared to be due to a standardization or calibration defect, whereas the biases in the upper region of the analytical measuring range were due to a marked underrecovery of 25(OH)D_2 in samples that contained both 25(OH)D_2 and 25(OH)D_3. This second defect makes the assay a poor choice for the evaluation of subjects taking vitamin D_2 as a supplement or receiving D_2 for the treatment of severe vitamin D deficiency. This defect also contributed to the overestimation of vitamin D deficiency in our clinical specimens. The manufacturer's limitation on the dilution and reanalysis of specimens with results greater than 96 ng/mL is further evidence that the assay is susceptible to matrix interference that can cause nonlinear dilution profiles in clinical specimens.

The differences in the types and magnitudes of the relative analytical errors observed for the group of samples that contained only 25(OH)D_3 and the group that contained both 25(OH)D_2 and 25(OH)D_3 proved that neither the Centaur2 nor the Architect is capable of providing accurate t25(OH)D results to laboratories whose workloads comprise a mixture of the 2 sample types. Our results show that a failure to quantitatively recover 25(OH)D_2 continues to be an important cause of intermethod variability in 25(OH)D analyses and that the use of either the Centaur2 or the Architect methods could result in an increase in the prevalence of vitamin D deficiency/insufficiency in the population being tested.

One potential limitation of our study is that an observed difference between a test method and a comparison method could have been caused by an analytical flaw in the comparison method. To minimize the possibility of falsely criticizing the performance of a test method, we used 2 independent comparison methods based on different analytical principles and focused our attention on significant analytical errors that were detected relative to both comparison methods. Furthermore, our findings of excessive random variability, the presence of standardization or calibration defects, over- or underrecovery of 25(OH)D_2, and matrix interference concur with those of other investigators who have recently evaluated the Centaur2 and the Architect methods.[30,31,39] Despite the growing evidence for the analytical limitations of these 2 methods, their use has increased steadily. Our review of the results from the College of American Pathologists Ligand Special Survey (sets Y-A and Y-B) showed that between March and September 2012, the sizes of the Centaur2 and Architect peer groups increased from 147 to 213 and from 41 to 81, respectively. This combination of questionable analytical quality and increased popularity suggests that these 2 new methods are likewise adding to the noise when it comes to building an evidence base that will improve our understanding of the roles of vitamin D in biology and medicine.

One barrier to the development of accurate and precise 25(OH)D methods is that the analyte is difficult to assay owing to its hydrophobic nature and ability to bind to lipids and proteins, including vitamin D binding protein; the presence in serum and plasma of variable ratios of 25(OH)D_2 and 25(OH)D_3; and the presence of multiple vitamin D metabolites that can cross-react in the immunoassays and coelute with 25(OH)D in the chromatographic methods.[17,18,40,41] Accuracy and precision can be further compromised by interfering proteins that affect IAs, matrix constituents that cause ion suppression in MS-based methods, and a variable recovery of t25(OH)D during sample extraction.[42] A second barrier is that some manufacturers have released finished products that have not addressed well-known analytical pitfalls of 25(OH)D analysis. For example, published studies from many different laboratories teach that a t25(OH)D assay used for patient care should accurately measure both 25(OH)D_3 and 25(OH)D_2,[43] demonstrate lot-to-lot consistency in reagent composition and quality,[44] be correctly standardized and calibrated,[45] and be free from matrix interference.[19] A third barrier has been the use of suboptimal protocols for the evaluation of candidate 25(OH)D methods by clinical laboratories. Herrmann[18] has recently presented 5 critical aspects of the analytic performance of a 25(OH)D method that are

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often overlooked during the design of method evaluation studies and during the review of analytical performance data that are reported in the literature or provided by manufacturers. A closer scrutiny of a candidate method relative to more rigorous criteria for acceptability would lead to an immediate improvement in the quality of patient test results and would eventually reduce intermethod variability as poorly performing methods are either modified by their manufacturers or withdrawn from the market.

As the number of available 25(OH)D methods rises to meet the increased demand for testing, it will be essential to carefully standardize and harmonize all available methods to enable the development and use of evidence-based clinical guidelines. Fortunately, the Vitamin D Standardization Program (VDSP) is making substantial progress toward both goals. Under its ongoing interlaboratory comparison study, 25(OH)D results determined by 36 participating laboratories (including 6 commercial labs and 16 assay manufacturers) for a set of 50 single-donor clinical specimens are being compared with results determined using the NIST-Ghent University RMPs for 25(OH)D.[14] The results of this study, which are scheduled to be publicly disclosed in the fall of 2013, are expected to reveal the strengths and limitations of the major methods that are now being used for patient testing. The knowledge gained from the VDSP should eventually transform test results measured by poorly performing assays from rough estimates of a patient's vitamin D nutritional status into standardized and harmonized results that will more precisely define a person's status relative to evidence-based reference values. In the meantime, clinicians and laboratories must become more aware of the limitations of current 25(OH)D methods and the negative impact that the current levels of inaccuracy and intermethod variability are having on the quality of patient care and on the value of using laboratory tests for the evaluation of vitamin D nutrition.

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