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Current Status of Clinical 25-hydroxyvitamin D Measurement: An Assessment of Between-Laboratory Agreement

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Abstract

Background—Historically, methodological differences and lack of standardization led to between-laboratory variability in 25(OH)D results. Recent observations raised concern about persisting variability. This quality assurance exercise investigated 25(OH)D result comparability between laboratories.

Methods—Serum pools (n = 25) were prepared to contain endogenous 25(OH)D₂ and 25(OH)D₃ at 25(OH)D concentrations from ~12–150 nmol/L (5–60 ng/mL). Aliquots were sent to 8 laboratories utilizing various 25(OH)D assay methods including high performance liquid chromatography with ultraviolet detection (LC-UV), LC with tandem mass spectroscopy detection (LC-MS/MS) or an automated immunoassay (Diasorin Liaison). The LC-UV results were selected as a referent to which all others were compared using linear regression and Bland-Altman analysis.

Results—Good correlation ($R^2 = 0.87$ to 0.97) was observed for all laboratories. Modest systematic bias was observed for some laboratories ranging from a positive mean bias of 10.5 nmol/L (4.2 ng/mL) to a negative mean bias of 3.5 nmol/L (1.4 ng/mL). For the laboratory with the greatest bias, 22/25 results were numerically higher (mean +15.7%) than LC-UV results. For Liaison, the primary error was likely random, whereas the major LC-MS/MS assay error source were biases likely due to calibration issues.

Conclusions—Modest inter-laboratory variability persists in serum 25(OH)D measurement. The National Institute of Standards & Technology 25(OH)D Standard Reference and calibration materials will further improve between-laboratory agreement for chromatography-based assays.

Keywords

Vitamin D; 25-hydroxyvitamin D; measurement; ergocalciferol; cholecalciferol

Introduction

Suboptimal vitamin D status is extremely common due to low dietary intake and 21st century lifestyle that limits exposure to sunlight [1–4]. Deficiency of vitamin D has classically been associated with bone disease and muscle weakness [5–7]. More recently,

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local effects of vitamin D have been suggested and associational studies find vitamin D deficiency to be associated with increased risk of a multitude of diseases including diabetes, cancer, various infections and vascular disease [8].

Measurement of circulating 25(OH)D concentration is accepted as the best clinical indicator of an individual's vitamin D status [9]. As such, 25(OH)D measurement is increasingly being obtained by clinicians who utilize this value to assess a patient's vitamin D status, and subsequently make decisions regarding supplementation or use of high-dose prescription vitamin D. However, in the recent past, substantial between-laboratory variability has been present [10-13]. In part, this variability has reflected differing methodologies utilized by various laboratories.

Briefly, 25(OH)D assays can be categorized into two basic groups; immunoassay methods that use antibodies directed against the D₂ and/or D₃ forms of 25(OH)D or chromatographic methods that separate 25(OH)D₂ and 25(OH)D₃ based on their respective chemical properties. In our opinion, there are advantages and disadvantages of both approaches. For example, immunoassays are more easily automated and provide short turnaround time with user-friendly equipment available at low cost. In contrast chromatographic methods are more complex, may have longer turnaround time and require skilled personnel using higher cost equipment. Additionally, immunoassays are capable of measuring only total 25(OH)D and are subject to matrix issues whereas chromatographic methods can quantify both 25(OH)D₂ and 25(OH)D₃ with high precision. Finally, immunoassays require secondary standards (calibrators) whereas chromatographic methods can utilize primary standards. However, it appears that Diasorin users have an advantage in that their calibrators are from a common source.

Immunoassay 25(OH)D procedures are often selected by clinical laboratories based on the advantages noted above. Chromatographic assays, especially when coupled with mass spectrometry are becoming the preferred method for high volume reference laboratories that can justify the greater instrument costs yet provide greater specificity and deliver quantitative values for both 25(OH)D₂ and 25(OH)D₃. Consistent with this, both the Center for Disease Control (CDC) and NIST have established LC-MS/MS 25(OH)D methods for use in their laboratories.

As both immunoassay and chromatographic based methods are in widespread clinical use, evaluation of result comparability between laboratories using these approaches is necessary. The purpose of this quality assurance exercise was to evaluate the current agreement of 25(OH)D measurement in U.S. laboratories.

Materials and Methods

QA Serum Specimens

De-identified residual serum samples collected from the UWHC routine 25(OH)D workload, and from quality assurance pools at the University of Wisconsin Osteoporosis Clinical Research Program, were selected on the basis of their 25(OH)D₂ and 25(OH)D₃ content. Twenty-five serum pools were prepared with the objective of obtaining samples contain varying concentrations of endogenous 25(OH)D₂ and 25(OH)D₃ (as measured by UWHC LC-UV) and also to span a clinically relevant range from ~ 12-150 nmol/L (5-60 ng/mL). The goal was to have serum pools with predominately 25(OH)D₂, a second set predominately 25(OH)D₃ and a third set with a mixture of both 25(OH)D₂ and 25(OH)D₃. Of these pools, 16/25 contained measurable amounts of 25(OH)D₂ (Figure 1). Aliquots (1.2 mL) of these serum pools were prepared and frozen at -70° C until shipped to the participating laboratories for analysis.

Clinical laboratories to which the authors or their colleagues send specimens for 25(OH)D measurement were identified. These laboratories used either the Diasorin Liaison Total 25(OH)D immunoassay or an LC-MS/MS method. Those laboratories that were interested in participating in this QA exercise were sent a single set of 25 serum aliquots on dry ice for 25(OH)D analysis. Fictitious “patient names” and “birthdates” were assigned to facilitate processing as routine clinical specimens.

25(OH)D Measurement

Serum 25(OH)D was measured in the serum pools by LC-UV ($n = 1$; UWHC), LC-tandem mass spectroscopy ([LC-MS/MS] $n = 3$) or chemiluminescent technology ([Diasorin Liaison] $n = 4$). These specimens were run on a single day in all participating laboratories. It is not known with certainty whether analyses of these specimens were conducted in duplicate by any of these laboratories. However, it is our experience that clinical specimens are not run in duplicate. The UWHC quantitates 25(OH)D with an extraction procedure that introduces an internal standard (lauraphenone) and isolates the 25(OH)D from the serum proteins. A solid phase extraction column further cleans the sample prior to quantitation by UV absorbance at 230 nm of the 25(OH)D₂ and 25(OH)D₃ constituents as they elute off the column [10]. The UWHC LC-UV assay uses commercial quality control material that contains a mixture of 25(OH)D₂ and 25(OH)D₃ at two different levels. At the lower concentration the method has demonstrated %CVs of 7.4 and 7.0 at 25(OH)D₂ and 25(OH)D₃ concentrations of 64.10 nmol/L (25.64 ng/mL) and 52.68 nmol/L (21.07 ng/mL), respectively. Using a higher concentration pool, the %CVs of this assay is 7.0% and 5.1% at 25(OH)D₂ and 25(OH)D₃ concentrations of 146.85 nmol/L (58.74 ng/mL) and 130.18 nmol/L (52.07 ng/mL), respectively. The UWHC laboratory participates in an international vitamin D proficiency-testing program (DEQAS) to judge this method versus other participating laboratories.

NIST Standard Reference Material (SRM) Measurement

The NIST SRM 972 [14] materials were assayed to judge how the UWHC LC-UV method compares to the NIST target values for both 25(OH)D₂ and 25(OH)D₃. These analyses were performed to provide an accuracy assessment of the UWHC method relative to the NIST reference materials.

Data Analysis

UWHC clinical LC-UV serum 25(OH)D results were arbitrarily defined as the referent to which the other results were compared using linear regression and Bland-Altman analyses (Analyse-it, Leeds, UK). The correlation coefficient (R^2) values, slopes and intercepts were obtained from the regression equations between the three LC-MS/MS methods along with the four Liaison chemiluminescent immunoassay methods compared to the UW LC-UV assay. These statistics provide insight as to how each of the methods compared to the UW LC-UV assay and where sources of discrepancies may have occurred. Vitamin D status was defined as “low” if the total serum 25(OH)D was < 75 nmol/L (30 ng/mL) [15].

Results

25(OH)D Data

Least squares statistics derived from analysis of the 25 serum pools by the seven laboratories using the UWHC LC-UV as the comparator are presented in Table 1. No outliers were present in these data; as a result, no individual 25(OH)D value was excluded. In comparison to the UWHC LC-UV results, good overall agreement was observed for all laboratories, based on correlation coefficients (R^2), slopes, intercepts and mean bias. Linear regression

and Bland-Altman plots comparing the LC-MS/MS and Liaison data to the UWHC LC-UV values are presented in Figures 2a and 2b respectively. The Bland-Altman analyses did not demonstrate substantial concentration-dependent bias; the greatest mean bias ranged from +10.5 nmol/L (4.2 ng/mL) to -3.5 nmol/L (1.4 ng/mL).

The four Liaison methods provided quite good agreement with the UWHC LC-UV assay based on their slopes, intercepts and correlation coefficients (Table 1). The four Liaison laboratories had slopes that were within 10% of the UWHC LC-UV method and three of the four Liaison laboratories had slopes of less than 5%. This general agreement between the four Liaison laboratories likely reflects their use of a common calibrator from the manufacturer.

Two of the three laboratories using LC-MS/MS methods exhibited the largest mean bias for 25(OH)D. The first two LC-MS/MS methods had slopes consistent with a positive bias of approximately 18% above the results from UWHC LC-UV assay. Thus, these two LC-MS/MS laboratories would report higher total 25(OH)D values than the UWHC LC-UV, such that at 75 nmol/L (30 ng/mL), their expected values would be close to 88.5 nmol/L (35.4 ng/mL). The third LC-MS/MS assay had a slope of 0.995 and intercept of 0.645 nmol/L (0.258 ng/mL), thus very closely agreeing with the UWHC LC-UV assay.

To evaluate whether the positive bias observed with the first two LC-MS/MS laboratories was shared equally between the measurement of 25(OH)D₂ and 25(OH)D₃, regression statistics were generated to compare LC-MS/MS results with the UWHC LC-UV data independently for 25(OH)D₂ and 25(OH)D₃ (Table 2). These regression statistics from the 25(OH)D₂ and 25(OH)D₃ results suggest that Laboratory 1 has a significant proportional error component based on a slope of 1.183 with 25(OH)D₂ while Laboratory 2 also has a significant proportional error component based on a slope of 1.213 with 25(OH)D₃. Thus, Laboratory 1 is capable of generating reliable total 25(OH)D data if the patient is not receiving high dose 25(OH)D₂ therapy and the reverse true for Laboratory 2 with reliable data only if the patient had a majority of their 25(OH)D as the D₂ form. LC-MS/MS Laboratory 3 has a slight positive slope for 25(OH)D₂ and a negative one for 25(OH)D₃.

To determine if there were any samples that exhibited an interference that might be consistent with random error, the results for each specimen were compared with the corresponding UWHC LC-UV result. For most of these specimens the Liaison results were close to the identity line or had values on both sides of the identity line. However, there were four samples in which all four Liaison results were biased either high, or low (examples highlighted in Figure 2b) with respect to the UWHC LC-UV result.

Clinical Assessment

If a 25(OH)D clinical decision level of 75 nmol/L (30 ng/mL) is applied to define “low” vs. “optimal” 25(OH)D status specimens containing “low” 25(OH)D concentrations are reproducibly low in all these laboratories. However, as with all “cutpoint” diagnostic approaches, individual specimens near this clinical decision level (here near 75 nmol/L [30 ng/mL]) are variably classified as “low” or “optimal” depending upon the laboratory (Figure 3).

NIST Standard Reference Material (SRM) Assessment

Good agreement was observed between the NIST-certified values on SRM 972 and those obtained by UWHC LC-UV ($R^2 = 0.978$). The UWHC LC-UV values compared to the NIST SRM targets are reported in Table 3.

Discussion

In this study, we find quite good agreement between two widely used 25(OH)D clinical measurement approaches; the Diasorin Liaison chemiluminescent immunoassay and LC-MS/MS. While there is good general agreement of 25(OH)D results, rigid application of a “cutpoint” diagnostic approach or clinical decision level (e.g., 30 ng/mL [75 nmol/L]) will continue to variably identify individuals, particularly those with values close to whatever decision level is selected, as being “low” or “optimal” depending on the laboratory. It is worthy of emphasis that imprecision is present in all quantitative tests. However, it is our experience that clinicians and patients may not adequately appreciate this variability and the resulting effect on a cutpoint-dependent diagnosis. In this regard, we have observed clinicians diagnose vitamin D deficiency based upon a 25(OH)D of 29 ng/mL (72.5 nmol/L) and prescribe high-dose ergocalciferol therapy. Clearly, a value of 29 ng/mL (72.5 nmol/L) is not different than 31 ng/mL (77.5 nmol/L), that might be considered to reflect optimal vitamin D status. Such clinician confusion regarding application of a cutpoint approach, combined with existing controversy surrounding the “optimal” 25(OH)D value[15;16] and the apparent safety of 25(OH)D concentrations in the 30-70 ng/mL range,[17;18] has led some to recommend that clinicians aim to achieve 25(OH)D values of ~40 ng/mL (100 nmol/L) in their patients.[19] Finally, but importantly, clinicians can have confidence that an individual “low” 25(OH)D value indicates vitamin D deficiency in their patient.

The 25(OH)D Liaison and LC-MS/MS measurement approaches are substantially different analytical methods. The Liaison uses antibodies to bind 25(OH)D that produces a chemiluminescent signal for quantitation. This antibody recognizes two different compounds, 25(OH)D₂ and 25(OH)D₃ with equal affinity. Thus, the Liaison by principle is an analysis of total 25(OH)D. In contrast, LC-MS/MS and LC-UV employ a chromatographic separation to isolate 25(OH)D₂ and 25(OH)D₃ and the MS/MS or UV system to detect and quantitate these compounds. Whether quantitation of both 25(OH)D₂ and 25(OH)D₃ has clinical relevance is debatable. It is currently accepted that total 25(OH)D measurement is appropriate to judge a patient's vitamin D status; as such, separate quantitation of 25(OH)D₂ and 25(OH)D₃ is not essential. However, if a patient is receiving high dose ergocalciferol (D₂), quantitation of 25(OH)D₂ can potentially reveal the degree to which the patient is compliant with treatment.

Information from the linear regression equations provides insight regarding differences between the UWHC LC-UV results and the individual methods; low R² values represents more random error between methods, differences in slopes are suggestive of proportional error between methods, and substantial intercepts can be attributed to constant error. Ideally, R² should be 1.0 and the slope and intercepts be 1.0 and 0.0 respectively for two methods to achieve perfect comparison. In our opinion, acceptable assay performance would be when the R² term is 0.95 and higher, with a slope of 1.0 ± 0.05 and an intercept of ± 5 nmol/L (two ng/mL). As the methodological principles employed for the immunoassay and chromatography assays are quite different, the sources and types of errors are also dependent upon the analytical scheme.

The four Liaison laboratories all had slopes above 0.87, suggesting that all were in reasonable agreement with respect to the UWHC LC-UV assay. In addition, all four had small and insignificant intercepts with respect to the UWHC LC-UV data. Since the Liaison assays are all calibrated with material supplied by Diasorin, there should be general agreement between their slopes and intercepts. However, these laboratories did have lower R² values relative to the UW LC-UV assay, which may reflect more random error.

It is not surprising that an immunoassay is subject to random error in that antibody binding can be influenced by slight changes in protein concentration, pH and ionic strength of the matrix in which the analyte is being measured. All immunoassays face challenges from the presence of interfering compounds and matrix issues. Interfering substances are fairly common and generally are compounds that share a similar chemical structure to the analyte being measured and thus bind to the antibody. Additionally, proteins or antibodies in a given patient sample can alter antibody binding thereby impacting the analytical value for a given specimen. These proteins or antibodies are not present in all patient specimens but when present can alter the patient's 25(OH)D result on a random basis. Such error is generally sample-specific and often not observed in every sample to the same extent. Thus, interference in immunoassays is likely to be a random occurrence.

The LC-MS/MS methods in general had less random error but two of three laboratories had substantial bias. Since the LC-MS/MS methods report both 25(OH)D₂ and 25(OH)D₃, we were able to generate regression equations for the two analytes separately and find that LC-MS/MS Laboratory 1 had the majority of its bias associated with 25(OH)D₂ while the Laboratory 2 bias was in 25(OH)D₃ measurement. Thus, these two LC-MS/MS assays both had significant calibration issues but differed in their origin. Clearly, the LC-MS/MS assays will benefit from the ethanol-based standards for 25(OH)D₂ and 25(OH)D₃ that are now available from NIST.

Chromatography has two areas of concern where analytical errors can occur. First is the calibration issue noted above; the recent availability of NIST ethanol-based standards should resolve calibration problems and allow laboratories to trace their calibrators to the NIST material. Secondly, the presence of interfering compounds is problematic when they elute at the same time as the analyte being measured. However, MS/MS detectors eliminate the vast majority of compounds that potentially co-elute either with 25(OH)D₂ or 25(OH)D₃ because the interfering substance would be required to have the same mass for the molecular ion being used to quantitate 25(OH)D₂ and 25(OH)D₃. The probability of an interfering substance having identical molecular ion mass is extremely low and is the reason that these methods offer the high level of specificity.

This study has limitations, most notably the relatively small sample size. This may have minimized the random error component observed with immunoassays as previously reported by Roth, et. al.[11] Overall, the current study suggests better agreement between laboratories that previously reported; [11;20] though this may reflect small sample size, it is plausible that automation with the Liaison system contributed to this observation. Additional limitations include that not all available 25(OH)D assay approaches were tested and the laboratories participating in this study were not randomly selected. Strengths of this study include the use of clinical laboratories, thereby facilitating the clinical applicability of these results, the selection of serum specimens containing both endogenous 25(OH)D₂ and 25(OH)D₃ and comparison with a chromatographic system that closely agrees with the NIST SRM results.

In conclusion, despite the above noted challenges for 25(OH)D measurement with either LC-MS/MS or Liaison 25(OH)D assays, there is overall good agreement such that these laboratories are capable of generating useful data to assess an individual's vitamin D status. Healthcare providers appreciate the arbitrary nature of all cutpoint diagnostic approaches; determination of an individual patient's vitamin D status is no different. The Liaison automated immunoassay has an important role to meet the high demand for 25(OH)D analysis for patient testing and is suitable for a general clinical laboratory. At the same time, the LC-MS/MS approach is well suited for the high volume commercial laboratories that seek high throughput and specificity that provides their clients with quantitation of both

25(OH)D₂ and 25(OH)D₃. It is apparent that laboratories must validate their calibration schemes and document their traceability to a national standard. The recent release of NIST ethanol-based 25(OH)D₂ and 25(OH)D₃ standards should facilitate this process.

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Figure 1.

Serum Pools Utilized in This Analysis. The serum pools selected for this evaluation were pre-defined by LC-UV measurement to span a clinically commonly observed 25(OH)D spectrum from “very low” to “high” as depicted here. Additionally, as the presence of 25(OH)D₂ might pose an additional challenge to accurate 25(OH)D determination, these pools were selected so that the majority contained measurable amounts of 25(OH)D₂, which also spanned the range from not detectable to “high.” Finally, six of these specimens had total 25(OH)D values near the common clinical decision level of 30 ng/mL (from 26.3 ng/mL to 34.7 ng/mL as determined by LC-UV).

Figure 2a

Figure 2b

Figure 2.

Agreement of 25(OH)D Results. Overall, good correlation exists between laboratories using LC-MS/MS (2a) or the Diasorin Liaison (2b). Systematic bias is observed with LC-MS/MS and LC-UV, likely reflecting calibration differences of the LC-MS/MS laboratories. Random error inherent in immunoassay approaches is suggested by samples that are uniformly “high” or “low” (e.g., grey filled symbols and black filled symbols respectively within the dashed rectangles) in the Liaison laboratories. Note: The smaller figures provide Bland-Altman plots for each individual laboratory compared to LC-UV results.

Figure 3.

Effect of 25(OH)D Result Variability on Clinical Decisions. When evaluated on a by patient basis, it is apparent that “very low” values (for example sample number 10) are in fact low, and “high” values are high, regardless of the laboratory in which the measurement is performed. Values near the often-used clinical decision level of 75 nmol/L (30 ng/mL) may be either above or below this “threshold” value.

Table 1**Least Squares Statistical Analysis Results**

Laboratory	R ²	Mean bias	Slope (95% CI)	p-value	Intercept (95% CI)	p-value
LC-MS/MS #1	0.97	+4.2	1.176 (1.085 to 1.267)	p < 0.0001	-1.176 (-4.27 to +1.91)	0.44
LC-MS/MS #2	0.95	+4.1	1.183 (1.068 to 1.298)	p < 0.0001	-1.52 (-5.419 to +2.381)	0.43
LC-MS/MS #3	0.91	+0.1	0.995 (0.857 to 1.133)	p < 0.0001	+0.258 (-4.413 to +4.929)	0.91
Liaison #1	0.90	-0.3	0.911 (0.778 to 1.044)	p < 0.0001	+2.381 (-2.130 to +6.893)	0.29
Liaison #2	0.87	-1.4	0.955 (0.795 to 1.116)	p < 0.0001	-0.073 (-5.503 to 5.358)	0.98
Liaison #3	0.93	+1.7	1.012 (0.895 to 1.129)	p < 0.0001	+1.284 (-2.675 to +5.243)	0.51
Liaison #4	0.89	-0.7	0.959 (0.804 to 1.113)	p < 0.0001	+0.547 (-4.302 to 5.397)	0.82

Note: 25(OH)D data reported as ng/mL

Table 2

Regression Analysis of 25(OH)D₂ and 25(OH)D₃ Results

Laboratory	25(OH)D ₂ Slope (95% CI)	p-value	25(OH)D ₂ Intercept (95% CI)	p-value	25(OH)D ₃ Slope (95% CI)	p-value	25(OH)D ₃ Intercept (95% CI)	p-value
LC-MS/MS #1	1.183 (1.122 to 1.245)	<0.0001	-0.742 (-1.934 to +0.449)	0.21	1.100 (1.044 to 1.156)	<0.0001	+0.828 (-0.391 to +2.048)	0.17
LC-MS/MS #2	1.069 (0.987 to 1.151)	<0.0001	-1.237 (-2.826 to +0.352)	0.12	1.213 (1.143 to 1.283)	<0.0001	+0.488 (-1.031 to +2.008)	0.51
LC-MS/MS #3	1.075 (1.020 to 1.131)	<0.0001	-0.152 (-1.231 to +0.928)	0.77	0.981 (0.850 to 1.112)	<0.0001	-0.363 (-3.213 to 2.487)	0.79

Note: 25(OH)D data reported as ng/mL

Table 3
UWHC LC-UV 25(OH)D Analysis of NIST SRM 972

Level	Analyte	NIST Certified Value ^{a&b}	UWHC LC-UV ^a
1 ^c	25(OH)D ₃	59.6 ± 2.1 (23.9 ± 0.8)	58.1 (23.5)
2 ^c	25(OH)D ₃	30.8 ± 1.5 (12.3 ± 0.6)	32.7 (13.1)
3	25(OH)D ₂	64.1 ± 4.8 (26.4 ± 2.0)	65.4 (27.0)
	25(OH)D ₃	46.2 ± 2.8 (18.5 ± 1.1)	51.7 (20.7)
4 ^{c & d}	25(OH)D ₃	82.3 ± 2.0 (33.0 ± 0.8)	77.6 (31.1)

^a Values reported in nmol/L (ng/mL)

^b Certified Values are weighted means from CDC and NIST analysis

^c Pool has 25(OH)D₂ below detection level of UWHC LC-UV method

^d Pool has 3-epi-25(OH)D₃ which is detected but not quantitated