Assay Variation Confounds the Diagnosis of Hypovitaminosis D: A Call for Standardization

N. BINKLEY, D. KRUEGER, C. S. COWGILL, L. PLUM, E. LAKE, K. E. HANSEN, H. F. DeLUCA, AND M. K. DREZNER

Department of Medicine Osteoporosis Clinical Center and Research Program (N.B., D.K., C.S.C., K.E.H., M.K.D.); Institute on Aging (N.B., D.K., C.S.C., K.E.H., M.K.D.); Geriatric Research Education and Clinical Center, William S. Middleton Veterans Affairs Medical Center (M.K.D.); and Department of Biochemistry (L.P., E.L., H.F.D.), University of Wisconsin-Madison, Madison, Wisconsin 53705

Endemic hypovitaminosis D contributes to osteoporosis development. However, variation in 25-hydroxyvitamin D (25OHD) measurement is reported and confounds the diagnosis of vitamin D insufficiency/deficiency. This report emphasizes the marked variability observed in serum 25OHD measurements between laboratories.

Initially, postmenopausal women had serum 25OHD determinations: 42 in laboratory A, 20 in laboratory B. Their mean (SEM) serum 25OHD concentrations were 46 (2.1) and 21 (2.3) ng/ml in laboratories A and B, respectively. Furthermore, there was little overlap in serum 25OHD among these clini- cally similar individuals. Specifically, 17% of those measured in laboratory A but 90% in laboratory B were below an arbitrary threshold value of 32 ng/ml.

Hypovitaminosis D is common due to low dietary vitamin D intake coupled with sun avoidance or minimal exposure (1–3). As a result, vitamin D inadequacy is a relatively recently recognized, but common, cause of secondary hyperparathyroidism with consequent bone loss and osteoporosis (4, 5). Furthermore, vitamin D insufficiency is associated with muscle weakness, and its correction reduces the risk for falls (6). Consequently, simply correcting this vitamin inadequacy should lead to improved skeletal health. Consistent with this, provision of supplemental vitamin D (7) or vitamin D plus calcium (8, 9) reduces fracture risk. Moreover, evidence is accumulating that vitamin D inadequacy may have other adverse health consequences, including increased risk of malignancies, hypertension, and diabetes mellitus (10, 11). Not surprisingly, much work has focused on defining the optimal circulating concentration of 25-hydroxyvitamin D (25OHD) and determination of the required intake to achieve this concentration. In this regard, expert opinion suggests that this serum concentration is approximately 80 nmol/liter (32 ng/ml) (12, 13). Thus, it is often recommended that clinicians strive to maintain 25OHD concentrations above 32 ng/ml in their patients to maximize bone health. Although previously noted (14–17), it is perhaps less widely appreciated that the assays used to measure 25OHD may yield discrepant results. This study highlights variability in 25OHD measurements obtained using current assays in different laboratories.

Subjects and Methods

Initial observation

Fifty-nine postmenopausal women were screened for potential participation in two osteoporosis clinical trials. A serum specimen from each woman was collected for 25OHD measurement; 42 samples were sent to laboratory A, and 20 were sent to laboratory B for analysis. Because three volunteers did not meet bone density criteria for the first study, they were subsequently screened for the second study.

Subsequent evaluation in healthy adults

Venous blood was obtained on April 8, 2003, from 10 healthy adult research staff and clinicians living in Madison, Wisconsin (~43° north latitude). Serum aliquots were prepared, and 20 ng/ml 25OHD (Amersham Searle, Arlington Heights, IL) was added to half of the aliquots (spiked specimen) from each volunteer. All aliquots were promptly frozen and maintained at –80°C until shipped for analysis. Spiked and unspiked aliquots from all individuals were sent to two clinical reference laboratories (C and D), two national central research laboratories (F and G), and two academic research laboratories (E and H) for 25OHD measurement. All laboratories were blinded to sample status, i.e. basal or spiked. Additionally, a local clinical laboratory measured serum calcium using a Cobas Integra 800 Autoanalyzer (Roche, Indianapolis, IN) and intact PTH with an immunochemiluminometric assay.

These studies were reviewed and approved by the University of Wisconsin Health Sciences Human Subjects Committee. Informed consent was obtained from all participants before any study-related procedure.

Vitamin D assay methodology

Table 1 lists the methods and normal range for 25OHD measurement in these laboratories.
TABLE 1. 25OHD assay methodology and normal range

<table>
<thead>
<tr>
<th>Laboratory</th>
<th>Methodology</th>
<th>Normal range</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>Acetonitrile extraction followed by in-house RIA</td>
<td>10–55 ng/ml</td>
</tr>
<tr>
<td>B</td>
<td>DiaSorin RIA</td>
<td>10–40 ng/ml</td>
</tr>
<tr>
<td>C</td>
<td>Acetonitrile extraction followed by DiaSorin RIA</td>
<td>8–38 ng/ml</td>
</tr>
<tr>
<td>D</td>
<td>Chemiluminescent assay</td>
<td>20–57 ng/ml</td>
</tr>
<tr>
<td>E</td>
<td>Acetonitrile extraction followed by DiaSorin RIA</td>
<td>NA</td>
</tr>
<tr>
<td>F</td>
<td>Chemiluminescent assay</td>
<td>6–54 ng/ml</td>
</tr>
<tr>
<td>G</td>
<td>Chemiluminescent assay</td>
<td>10–68 ng/ml</td>
</tr>
<tr>
<td>H</td>
<td>Ethyl acetate extraction followed by normal phase HPLC</td>
<td>NA</td>
</tr>
</tbody>
</table>

TABLE 2. Patient demographics for specimens sent to laboratories A and B

<table>
<thead>
<tr>
<th>Laboratory</th>
<th>N</th>
<th>Age (yr)</th>
<th>T-score</th>
<th>25OHD (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>42</td>
<td>64.0 (1.5)</td>
<td>-2.6 (0.1)</td>
<td>46.0 (2.0) [20–71]</td>
</tr>
<tr>
<td>B</td>
<td>20</td>
<td>67.2 (1.3)</td>
<td>-2.7 (0.1)</td>
<td>21.0 (2.3) [10–48]</td>
</tr>
</tbody>
</table>

Data represent mean (SEM). Range is given in brackets.

Statistical analysis

For the purpose of analysis, HPLC measurement of 25OHD (laboratory H) was established as the gold standard to which the results from other laboratories/assays were compared. Serum 25OHD measured in laboratories C–H was compared in basal and spiked specimens using factorial ANOVA with subsequent Fisher’s protected least significant difference testing using Statview software (Abacus Concepts, Cary, NC). Additionally, results of laboratories C–G were compared with laboratory H using Bland-Altman plots and Passing/Bablok regression analysis using Analyse-it software (Analyse-it Software Ltd., Leeds, UK).

Results

Demographics

Initial observation group. All of the subjects in both groups were Caucasian, postmenopausal women. Overall, their mean age was 65.0 ± 1.1 (SEM) yr (range, 49–86 yr). Osteoporosis was present in all of these women based on bone mineral density measurement at the lumbar spine and/or proximal femur using dual-energy x-ray absorptiometry. Their mean T score (lowest of the L1–L4 spine, total femur, femoral neck, or trochanter) was −2.7 ± 0.1. No differences were present in age, weight, or bone density between groups (Table 2).

Healthy laboratory personnel. All 10 individuals were Caucasian, and their mean age was 46.2 ± 3.7 yr (range, 25–57 yr). Serum calcium was normal in all (mean, 9.2 ± 0.1 mg/dl), but the intact PTH was elevated in two. Their mean serum PTH concentration was 37 ± 6.5 pg/ml and ranged from 13–73 pg/ml. The normal range in this laboratory is 7–53 pg/ml.

25OHD concentration/postmenopausal women

Despite the subjects in groups A and B being of similar age, geographic residence, and bone mass, there was almost no overlap in measured serum 25OHD concentration at these two national central laboratories (Fig. 1A). Additionally, if optimal serum 25OHD concentration is defined as being greater than 32 ng/ml, 90% of these women were insufficient at laboratory B, but only 17% in laboratory A. Furthermore, serum 25OHD concentration was measured in three women at both laboratories A and B with markedly differing results (Fig. 1B).

25OHD concentration/healthy laboratory personnel

In these 10 healthy adults, the mean serum 25OHD varied widely from 17.1 (4.6) to 35.6 (5.2) ng/ml (P < 0.005) between laboratories (Fig. 2A). Similarly, for a given subject, the 25OHD result was substantially different depending on the laboratory used (Fig. 2B). Interestingly, laboratories C and D are the sites to which clinical specimens are routinely sent in our area. As evident from these observations, whether a patient is considered as being vitamin D insufficient (using 32 ng/ml as a threshold) depends, in large measure, on the laboratory used. For example, subjects 1, 4, 6, 7, and 9 (Fig. 2B) would be classified as insufficient in some laboratories and normal in others. Thus, 50% of these individuals might be classified as having a different vitamin D status (insufficient or normal) depending solely on the laboratory used.

25OHD concentration: effect of spiking with 25OHD

In the spiked specimens, the mean serum 25OHD also varied (P < 0.05) between laboratories from 27.4 (4.5) to 44.6
Furthermore, the increase observed upon spiking with 20 ng/ml 25OHD was less than anticipated, e.g., from 7.7–18.0 ng/ml with the greatest increment detected by HPLC (Fig. 3). Furthermore, the measured increment produced by addition of a known quantity of 25OHD ranges from 17–95% of that expected, depending on the laboratory. Nevertheless, one facility measures 25OHD with excellent correlation to values obtained by HPLC and accurately measures the increment produced on sample spiking. Thus, accurate measurement of 25OHD is feasible in a commercial laboratory.

**Evaluation of results obtained in different laboratories**

When compared with HPLC, all laboratories (with the exception of laboratory E) demonstrated a highly significant correlation when evaluated by Bablock regression analysis (Fig. 4). However, significant positive bias was observed in the basal samples measured in laboratories D, F, and G (Table 3 and Fig. 4). In the spiked specimens, significant positive bias was observed only in laboratory D (Table 3 and Fig. 4).

**Discussion**

This study illustrates that putative advances in methodology for assessment of vitamin D status have not eliminated the previously reported substantial laboratory variation in serum 25OHD measurement (15, 18, 19). Indeed, the mean serum 25OHD concentration in 10 subjects differs 2-fold between laboratories, and the proportion of subjects below an arbitrary threshold of insufficiency (32 ng/ml) (12) varies from 17–90%. Furthermore, the measured increment produced by addition of a known quantity of 25OHD ranges from 17–95% of that expected, depending on the laboratory. Nevertheless, one facility measures 25OHD with excellent correlation to values obtained by HPLC and accurately measures the increment produced on sample spiking. Thus, accurate measurement of 25OHD is feasible in a commercial laboratory.

Vitamin D deficiency classically causes impairment of skeletal mineralization, resulting in rickets and osteomalacia in children or osteomalacia alone in adults. However, less severe degrees of vitamin D insufficiency contribute to bone loss without necessarily impairing bone mineralization. Specifically, vitamin D inadequacy leads to calcium malabsorption with resultant secondary hyperparathyroidism leading, over time, to bone loss and osteoporosis (5). In this context, optimal vitamin D status is defined as the serum 25OHD concentration above which PTH is not further suppressed. Applying this definition, hypovitaminosis D is extremely common due to low dietary intake and minimal cutaneous sunlight exposure (4, 13, 20–24). Additionally, vitamin D supplementation increases calcium absorption, improves bone density, reduces risk of falls, and decreases the likelihood of osteoporotic fracture (6, 8, 25–28). Finally, nonskeletal adverse effects related to inadequate vitamin D status, including such diverse consequences as hypertension, diabetes mellitus, cancer, and autoimmune disease, are receiving increased attention (29–32). As a result, there is an appropriately increasing emphasis on the measurement of 25OHD in the clinic and subsequent supplementation to maintain or enhance health status by attaining an optimal vitamin D status. In this regard, Chapuy et al. (13) and Heaney (12) have suggested that the optimal 25OHD serum concentration is above approximately 31–32 ng/ml (78–80 nmol/liter). However, our data indicate that widespread clinical application of a single target value, e.g., 32 ng/ml, is not appropriate at this time due to marked interlaboratory
variability. Thus, practicing clinicians do not have a reliable tool to detect hypovitaminosis D in their patients.

It is not surprising that discordant results are obtained because these laboratories use substantially different methodology for measurement of 25OHD, which, to this time, has not been standardized. For example, the chemiluminescent assay is, in essence, a protein binding assay that uses a reagent to separate vitamin D from its binding proteins without preparative chromatography (33). Thus, this procedure has similarities to protein binding assays that have been used for some time (34–36) and usually (14, 15, 37), but not always (17, 38), produce higher 25OHD results than other methodologies, perhaps reflecting the absence of a prior chromatographic separation (39). It is important to recognize that the competitive protein binding assays may use different sources of vitamin D binding proteins and modify the extraction or preliminary purification procedures and/or incubation conditions (33, 40). Thus, not all competitive protein binding assays are identical. Subsequently, RIA methodology using antibodies was developed (41) and documented to compare very well with an HPLC gold standard (42, 43). However, it is important to recognize that not all RIA assays are the same; some antibodies recognize both 25OHD\(_2\) and 25OHD\(_3\), whereas others underestimate 25OHD\(_2\) (16). Furthermore,
the fact that an RIA, which has previously been reported to correlate well with HPLC, performed variably in this study likely indicates that this procedure is user-dependent and requires rigorous quality control to ensure reliable results. Finally, HPLC has long been recognized as the gold standard (44–46); however, despite recent advances to improve its clinical applicability (47), it is not widely available to clinicians. We recognize that assay standardization is not a simple or inexpensive endeavor (48). However, given what appears to be an epidemic of hypovitaminosis D, the simplicity and low cost of its correction, and the potential beneficial skeletal and nonskeletal consequences of doing so, it is essential that the medical community define a threshold for optimal vitamin D status using accurate, reproducible assays. These assays must subsequently be internationally standardized and made available to practicing clinicians.

Limitations of this study include the small sample size and the fact that not every available methodology for 25OHD measurement was evaluated. However, these limitations do not negate the conclusion that widespread clinical application of a single threshold value to define optimal vitamin D status is impossible at this time.

In conclusion, marked between-laboratory variability exists in serum 25OHD measurement. As a result of this variability, an arbitrary definition of optimal serum 25OHD concentration is neither possible nor widely applicable. International standardization of 25OHD measurement is essential before elimination of endemic hypovitaminosis D is achievable. Given increased recognition of this situation by the National Institutes of Health (49), such standardization, perhaps through agencies such as the National Institute of Standards and Technology, seems attainable in the near future. In the interim, it is prudent that clinicians recommend routine vitamin D supplementation but, because of the difficulties inherent in measuring 25OHD, refrain from using a single 25OHD value to define optimal vitamin D status. Furthermore, the amount of supplemental vitamin D required remains controversial and likely varies with several factors, including age and severity of extant vitamin D deficiency. Although the recommended daily intake remains at 400–600 IU in the United States, recent data and, consequently, expert opinion suggest that substantially more (~1000 IU) may be required. Ideally, these higher doses should be monitored using an accurate 25OHD assay. Based on our study and those of others, clinicians should consider using RIA for 25OHD measurement to assess vitamin D status and response to supplementation until other methodologies are further validated.

### TABLE 3. Bias (nanograms/milliliter) observed when comparing laboratories with HPLC

<table>
<thead>
<tr>
<th>Laboratory</th>
<th>Basal sample</th>
<th>Spiked sample</th>
</tr>
</thead>
<tbody>
<tr>
<td>C</td>
<td>0.7 (−1.6 to 3.0)</td>
<td>−1.1 (−8.1 to 6.0)</td>
</tr>
<tr>
<td>D</td>
<td>18.5 (11.4 to 25.6)</td>
<td>9.4 (2.3 to 16.5)</td>
</tr>
<tr>
<td>E</td>
<td>2.6 (−6.6 to 11.8)</td>
<td>−7.9 (−21.5 to 5.8)</td>
</tr>
<tr>
<td>F</td>
<td>9.8 (6.6 to 13.9)</td>
<td>2.0 (−4.8 to 8.8)</td>
</tr>
<tr>
<td>G</td>
<td>9.9 (6.3 to 13.5)</td>
<td>3.0 (−2.8 to 8.6)</td>
</tr>
</tbody>
</table>

Data presented as bias (95% confidence interval).
Acknowledgments

Received November 14, 2003. Accepted February 24, 2004.

Address all correspondence and requests for reprints to: Neil Binkley, M.D., 2870 University Avenue, Suite 100, Madison, Wisconsin 53705. E-mail: nbinkley@facstaff.wisc.edu.

References

10. Zittermann A 2003 Vitamin D in preventive medicine: are we ignoring the evidence? Br J Nutr 89:552–572
44. Stenman UM 2001 Imprecision standardization: is it possible, who is responsible, who is capable? Clin Chem 47:815–820

JCEM is published monthly by The Endocrine Society (http://www.endo-society.org), the foremost professional society serving the endocrine community.