

Clinical Applications for Vitamin D Assays: What Is Known and What Is Wished for

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Vitamin D is a “hot topic,” with the number of citations in PubMed exceeding 2400 in 2009, a 3-fold increase in 1 year. In the US, the number of requested 25-hydroxyvitamin D (25-OHD)⁹ assays is increasing exponentially. Not all of the published material has validity, however. A panel of experts was invited to address a series of questions pertaining to laboratory methods and clinical applications of available assays for 25-OHD and 1,25-dihydroxyvitamin D (1,25-OHD).

What should we measure: 25-OHD₃, 25-OHD₂, both, or 1,25-OHD?



Rosemary L. Schleicher:

Our interest is in providing 25-OHD data for the National Health and Nutrition Examination Survey (NHANES). Separate estimates for 25-OHD₂ and 25-OHD₃—together with recent food- and supplement-intake data, questionnaire data about physical activity, sun-protection behavior and skin type, and demographic information related to race/ethnicity, sea-

son, latitude, and age—provide valuable information about the sources of vitamin D for those in the noninstitutionalized civilian US population. In addition, we will be separating and quantifying the C3 epimer of 25-OHD₃, which may not be as biologically active as 25-OHD₃.



John Eisman: 25-OHD₂ and 25-OHD₃ should be measured in most clinical situations, although in many countries vitamin supplements and food fortification are moving from vitamin D₂ to D₃. I am unaware of clinical needs requiring knowing 25-OHD₂ and 25-OHD₃ separately, or the C3 epimer. There are few clinical situations where knowing the

1,25-OHD concentrations are clinically critical or overly helpful.



Roger Bouillon: We need to know the combined concentration of 25-OHD₃ and 25-OHD₂ because both products can be converted into the active hormone 1,25-OHD. In countries where vitamin D₂ supplementation is not available, the measurement of 25-OHD₃ alone would be sufficient, since there is very little vitamin D₂ in

natural nonsupplemented food, and this is the case for most European countries. In countries where vitamin D₂ is readily used, either as food additive or as dietary supplement, the 25-OHD₂ concentration should be included in the measurement. There are no solid clinical

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⁹ Nonstandard abbreviations: 25-OHD, 25-hydroxyvitamin D; 1,25-OHD, 1,25-dihydroxyvitamin D; NHANES, National Health and Nutrition Examination Survey; PTH, parathyroid hormone; MS/MS, tandem mass spectrometry; UV, ultraviolet; LC-MS/MS, liquid chromatography–MS/MS; VDR, vitamin D receptor; IOM, Institute of Medicine.

reasons to measure 25-OHD₂ and 25-OHD₃ separately. The desired assay would be one that recognizes equally 25-OHD₂ and 25-OHD₃ to provide total 25-OHD. Alternatively, there is a method that allows the measurement of both components, which then can be summed. Separate measurements may answer specific research questions.

Progressive kidney failure is the main reason for low 1,25-OHD concentrations, but its clinical usefulness in this disease is not yet settled. Measurement of serum 1,25-OHD may be helpful in assessing the origin of abnormal calcium concentrations, especially hypercalcemia. This measurement is essential for diagnosing hypercalcemia due to excess extrarenal 1,25-OHD production, such as in sarcoidosis or inflammation-driven hypercalcemia. Serum 1,25-OHD is slightly increased in hypercalcemia due to parathyroid hormone (PTH) excess, whereas it is decreased in most other non-PTH-related causes of hypercalcemia.



Ravinder J. Singh: It really depends on the differential diagnosis. For nutritional deficiency, definitely both forms, vitamin D₂ and D₃, should be measured. The liver converts vitamin D₂ and D₃ rapidly into 25-OHD₂ and 25-OHD₃, which are converted to 1,25-OHD₂ and 1,25-OHD₃, respectively,

in a calcium- and PTH-based negative-feedback loop. Since 25-OHD₃ and 25-OHD₂ circulate in ng/mL (nmol/L) concentrations, it is easier to develop methods to measure them than 1,25-OHD₂ and 1,25-OHD₃ metabolites (pg/mL, pmol/L). Most studies have measured only 25-OHD₃ and 25-OHD₂ and demonstrated associations with them. 1,25-OHD₂ and 1,25-OHD₃ are measured in renal-failure patients and patients with sarcoidosis and other granulomatous diseases.



Michael F. Holick: To determine a person's vitamin D status, it is important to know the total blood concentration of 25-OHD, which includes 25-OHD₂ and 25-OHD₃. In the United States, the only pharmaceutical prescription form of vitamin D is vitamin D₂, and therefore measuring 25-OHD₂ can be of great value in subjects treated for vitamin D deficiency. If the

patient's 25-OHD₂ does not increase, this could be due to noncompliance or a silent intestinal malabsorption syndrome, such as celiac disease.

The assay for 1,25-OHD should not be used to determine a person's vitamin D status, since patients with vitamin D deficiency and secondary hyperparathyroidism often have normal or increased concentrations of 1,25-OHD. However, this assay is of great value in evaluating the differential diagnosis for a variety of inborn and acquired disorders of calcium, vitamin D, and bone metabolism. It is especially valuable in patients with chronic kidney disease, primary hyperparathyroidism, sarcoidosis, oncogenic osteomalacia, vitamin D-resistant rickets, pseudo-vitamin D deficiency rickets, and hypophosphatemic rickets.

Is there a preferred assay for the measurement of vitamin D metabolites?

Rosemary L. Schleicher: We definitely prefer chemistry-based assays in which the different vitamin D metabolites are separated. For quantification, we prefer tandem mass spectrometry (MS/MS) using stable isotope-labeled internal standards to correct results for recovery. In theory, isotope dilution provides the best match of materials for correcting results. However, others have shown that ultraviolet (UV) detection provides results that are comparable with those from mass spectrometry. For NHANES, the availability of serum is limited because so many clinical tests are performed on each participant's blood. Mass spectrometry is more sensitive than UV detection, and thus it suits our needs best.

Ravinder J. Singh: Historically, various assays have been used, including RIA, vitamin D-binding protein assays, HPLC-UV, and liquid chromatography-MS/MS (LC-MS/MS) methods. Every method has advantages and disadvantages, and it is impractical for every laboratory to perform the reference method. It is important, though, for the assays to be standardized and harmonized. It took 50 years to achieve the standardization for cholesterol measurements, but until this is achieved for vitamin D testing, the value of the various published studies that used current assays is limited. Unfortunately, no critical error limits have been defined for vitamin D testing for making either clinical- or analytical-performance decisions.

Michael F. Holick: My assay of choice for measuring 25-OHD is LC-MS/MS, which quantitatively measures circulating concentrations of both 25-OHD₂ and 25-OHD₃, which then gives the total 25-OHD concentration. Many of the chemiluminescence assays use antibodies to measure 25-OHD in unextracted serum. This

is problematic since these antibodies recognize other vitamin D metabolites, such as 24,25-OHD, and therefore the assay not only provides the total 25-OHD concentration but also includes other 25-OHD metabolites. In addition, from my clinical experience I have found that these assays do not always recognize 25-OHD₂ and 25-OHD₃. Thus, patients being treated with vitamin D₂ for vitamin D deficiency may be told by their physician that they are not responding to therapy since their plasma total 25-OHD did not increase, when in fact it did. The CDC and the NIH are now using the LC-MS/MS assay as the preferred assay for clinical trials.

How stable are 25-OHD and 1,25-OHD during transport?

Rosemary L. Schleicher: The 25-hydroxylated metabolites that we measure are very stable in serum. When serum was kept unfrozen at temperatures up to 37 °C for over a week, the concentrations of 25-OHD₂, 25-OHD₃, or epi-25-OHD₃ were unchanged. In our lab, freeze–thaw data show excellent stability of these 3 analytes for at least 5 cycles.

John Eisman: The 25-OHD metabolites are very stable, as is 1,25-OHD in serum. However, they are not so stable once they have been extracted.

Roger Bouillon: According to published data confirmed by personal unpublished data, vitamin D metabolites are very stable at –20 °C and after freeze–thawing cycles. They are also stable for 24 h or so at room temperature.

Ravinder J. Singh: The concentration of 25-OHD₃ in its natural state bound to vitamin D–binding protein is very stable at room temperature, even for unprocessed whole blood. For either processed or unprocessed samples, delays of several hours before analysis had negligible effects on concentrations. Even “forgotten” samples or those received in an unfrozen state appear to be suitable for analysis. The decreases in concentrations noted after 3 days under usual laboratory conditions at room temperature were less than the analytical interassay imprecision. There appears to be no need for serum to be frozen for transport, and whole blood might even be the specimen of choice for transport for up to 3 days. Storage conditions of serum at 4 °C for at least 7 days and up to 4 freeze–thawing cycles are permissible. 25-OHD₃ concentrations seem to be stable at room temperature and under the common preanalytical conditions experienced in medical laboratories.

What are the clinical indications for measurement of 25-OHD in disorders of bone and mineral metabolism, or other situations?

John Eisman: Our experience, supported by other international studies and by using appropriate assays, is that 25-OHD concentrations are quite low in many people with osteoporosis and in many otherwise healthy people.

Michael F. Holick: We have developed LC-MS/MS and HPLC assays for vitamin D that have been valuable in evaluating the role of UV irradiation on raising blood concentrations of vitamin D. They have also been of value in determining the degree of vitamin D malabsorption in patients with inflammatory bowel disease, cystic fibrosis, and ulcerated colitis, and after gastric bypass surgery.

All Experts: In other situations, there is no need for specific measurement of vitamin D itself for clinical purposes outside of a research setting that addresses a question about the origin or metabolism of vitamin D itself, or population studies. The many epidemiologic studies linking vitamin D deficiency to specific conditions/diseases or for predicting adverse health outcomes are intriguing but as yet of little clinical relevance.

What reference ranges should be used for reporting 25-OHD results, and should they be stratified by sex, ethnicity, age, and season?

Rosemary L. Schleicher: For population surveys, we are interested in demographic differences. Our data from NHANES show that there are differences between the sexes (2% difference between males and females), in race/ethnicity (67% difference between non-Hispanic whites and non-Hispanic blacks), in age (33% difference between young children and the elderly) and in season/latitude (8% difference between winter in the south and summer in the north).

John Eisman: Measuring the values in a population and presuming this is what should be is as rational as accepting significant obesity, hypertension, diabetes, renal impairment, and osteoporosis as being “normal.” In the absence of any data on what is an age-, sex-, ethnicity-, or latitude-appropriate concentration, I believe we have to use only the seasonal data. Someone with a borderline 25-OHD at the end of winter may be fine during summer and thus probably overall throughout the year. Someone whose 25-OHD is borderline at the end of summer will almost certainly be low in winter and thus deserves care and consideration

of replacement and investigation to exclude other causes, such as celiac disease.

Roger Bouillon: 25-OHD should be higher than 10 $\mu\text{g/L}$ (25 nmol/L), because such persistently low concentrations may result in impaired mineral deposition (rickets/osteomalacia). In adults, 25-OHD should be higher than 20 $\mu\text{g/L}$ (50 nmol/L) to avoid compensatory mechanisms on PTH secretion or calcium absorption, and thus bone balance. Values are affected by season and sunlight exposure, skin pigment, and ethnicity.

Ravinder J. Singh: It is clear that critical clinical cutoff values will be different for patients at different latitudes, races, and diseases. For example, prevention of rickets in neonates and bone loss in adults will have very different cutoff values. For diseases such as diabetes and cardiovascular disease, it will be very hard to determine cutoff values, since these diseases are complex disorders and being multifactorial will have broad reference range values in these populations.

Michael F. Holick: I do not believe that a plasma 25-OHD concentration should be based on sex, ethnicity, age, or season. I believe that all children and adults should maintain a plasma concentration of 25-OHD of at least 30 $\mu\text{g/L}$ (75 nmol/L) and up to 100 $\mu\text{g/L}$ (250 nmol/L) is safe. The only exception is in patients with extrarenal production of 1,25-OHD, including patients with chronic granuloma disorders and some lymphomas.

How important are sunscreens and sun hats in regulating synthesis of vitamin D?

Rosemary L. Schleicher: Sunscreens and clothing effectively prevent cutaneous synthesis of vitamin D. In NHANES, total serum 25-OHD was significantly higher in those who often or sometimes practiced sun-protection behaviors, compared with those who did not.

John Eisman: In one study from Victoria, Australia, sunscreen use in a randomized clinical trial was not associated with significant differences in 25-OHD concentrations. That may relate to compliance and adoption of other sun-safe practices in Australia.

Roger Bouillon: Both protections will decrease vitamin D production by UVB radiation and 25-OHD concentration.

Ravinder J. Singh: It depends whether the source of vitamin D is the supplementation or sun exposure.

Michael F. Holick: If the sunscreen is used properly, it will markedly reduce the synthesis of vitamin D in the skin. For example, a sunscreen with an SPF of 30 by definition should absorb approximately 98% of incident UVB radiation and thus will reduce the ability of the skin to produce vitamin D by approximately 98%. I always encourage sunscreen use on the face and the use of a sun hat to protect the face from damaging effects from excessive exposure to sunlight. Since the face is the most sun-exposed area of the body, it is most prone to sun damage and increased risk for nonmelanoma skin cancer. Also, it represents only about 9% of the body surface and thus provides very little vitamin D. For all these reasons, when one is exposed to sunlight, some type of sun protection of the face is encouraged.

How valid are the epidemiologic data relating vitamin D to diseases outside the skeleton?

John Eisman: There are interesting data, but it is not possible to disentangle health status from lifestyle and thus to establish causal relationships.

Roger Bouillon: On the basis of cross-sectional and to a limited extent prospective epidemiologic data, low vitamin D status is usually an indicator of poor health status or outcome. Much of the epidemiologic data provide conflicting information. Most randomized controlled studies report no net beneficial effects on extraskeletal tissues.

Michael F. Holick: There has been a lot of discussion and debate about association studies and observational studies that have suggested that living at higher latitudes and therefore being at higher risk for vitamin D deficiency increases risk of autoimmune diseases, cardiovascular disease, type II diabetes, infectious diseases, preeclampsia, cesarean section, and deadly cancers. Taken as a whole, the data are very credible and clearly worthy of further investigation. There are now several randomized controlled trials demonstrating that enhanced vitamin D intake reduces risk of asthma attacks, vascular smooth muscle stiffness, influenza, and infection, and improves insulin sensitivity, findings that help support many of the claims made by association and observational studies.

What are the mechanisms controlling 1,25-OHD production outside the kidney?

John Eisman: There is good evidence for extrarenal production of 1,25-OHD in skin and hematopoietic tissues, among others. It is likely in these sites that 1,25-OHD exerts some paracrine regulatory role. Many regulatory molecules have been invoked as being impor-

tant in regulation of extrarenal 1,25-OHD. However, it is clear that there is a loss of negative feedback from 1,25-OHD and no evidence of regulation by PTH in those sites.

Roger Bouillon: Extrarenal synthesis is regulated by factors other than those in the kidney, which are best documented in the monocyte and immune system and, to a lesser extent, in keratinocytes.

Michael F. Holick: It is now well documented that activated macrophages have the ability to produce 1,25-OHD, which plays an important role in helping the cell to destroy infective agents, such as tuberculosis, by enhancing the production of cathelicidin. We, as well as many other investigators, have demonstrated that human keratinocytes, colonic cells, and prostate cells, among others, express the enzyme that is capable of producing 1,25-OHD. More research is needed to understand how important the local production of 1,25-OHD is for regulating cell growth and a wide variety of other gene activities.

Is there a role for determining polymorphisms in the vitamin D receptor (VDR)?

John Eisman: This is still a developing area in the sense that polymorphisms in the VDR, among those in many genes, will eventually play a role in pharmacogenetics in relation to choices of therapy. However, we are not there yet. VDR polymorphisms have been reported to have an effect on bone phenotypes in many studies and do appear on the lists of possibly associated genes in the large genome-wide association studies.

Roger Bouillon: The impact of VDR polymorphism is not great and certainly not uniform in different populations, but minor effects cannot be excluded and are even likely—so no clinical value at present.

Ravinder J. Singh: Since large numbers of patients are being treated with higher doses of vitamin D, it will be great to have the knowledge of not only the polymorphisms of the VDR but also the genes involved in pharmacogenomics.

Michael F. Holick: The literature on VDR polymorphism has a checkered history. Originally, it was thought that the major cause of osteoporosis was due to a VDR polymorphism. However, this turned out not to be true. There does appear to be some association with other VDR polymorphisms and increased risk for chronic diseases, including prostate cancer. More research is needed to better define the cause–effect relationship of VDR polymorphism in chronic diseases.

There is mounting evidence that the polymorphism for the vitamin D–binding protein may play a very important role in determining a person's vitamin D status. This needs further investigation.

In an era of shrinking resources in healthcare and given the existing clinical and population studies, do you support the measurement of vitamin D in general practice in subjects with no clinical signs or suspicion of bone disease?

John Eisman: Vitamin D insufficiency is common in otherwise healthy people around the world, even near the equator, and is likely more common in those individuals with dark skin, those with little skin exposure (often culturally determined), and those who have limited sunlight exposure, e.g., related to work commitments. In such individuals, a measurement of 25-OHD, particularly at the end of winter, provides clarity about their precise situation and whether or not they might benefit from long-term vitamin D replacement. This is critical in those with any suggestion or evidence of malabsorption or bone disease.

There needs to be more careful consideration of what might be optimum 25-OHD concentrations. I believe the recent Institute of Medicine (IOM) report has swung the pendulum too far away from the other extreme of advocating very high values. In my opinion, neither is supported by very good evidence. To avoid the “fine in summer but too low in winter” situation, it would seem that aiming for closer to 30 $\mu\text{g/L}$ (75 nmol/L) would be sensible. It would probably be adequate in winter and unlikely to produce any toxicity issues in summer.

Roger Bouillon: A serum measurement should indeed be performed in general practice only when the result has diagnostic or therapeutic implications. For an otherwise healthy (North American) population, vitamin D supplementation should be on the order of 400–800 IU/day, depending on age, according to a recent IOM 2010 statement. If this is applied in practice, then 25-OHD concentrations will be $>20 \mu\text{g/L}$ (5 nmol/L) in most normal subjects, and additional measurement of 25-OHD is not needed. So, routine screening of vitamin D deficiency as part of a general health evaluation is not recommended. As long as there is no formal proof that higher-than-normal vitamin D supplementation has extraskeletal effects, it is doubtful whether 25-OHD measurements have real practical implications.

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