A True Assessment of Total Vitamin D
Measuring Both 25(OH) Vitamin D2 and D3
Introduction
Globally, over one billion people are vitamin D deficient, and in the United States 77% of U.S. adults are insufficient. Increases in vitamin D testing can be attributed to a growing global deficiency due to limited sun exposure and increasing links between vitamin D deficiency levels and health conditions. In order to ensure that laboratories are providing accurate testing results, it’s important their vitamin D testing methodology measures total vitamin D (25(OH) vitamin D, and D3), is traceable to LC-MS/MS, and has acceptable precision.

Vitamin D
1,25(OH)2 vitamin D is a steroid hormone that plays a critical role in intestinal absorption of calcium and maintaining calcium homeostasis. It has a major role in forming and maintaining strong and healthy bone. This steroid hormone is produced by two sequential enzymatic hydroxylation steps of vitamin D. The first takes place in the liver, forming 25(OH) vitamin D. This metabolite is then carried to the kidney where the second enzymatic step forms 1,25(OH)2 vitamin D.

Because 1,25(OH)2 vitamin D is closely regulated by parathyroid hormone (PTH) and intestinal calcium, this form of vitamin D often does not accurately reflect vitamin D status. This steroid hormone form of vitamin D circulates at extremely low concentrations, making it more difficult to measure accurately. Vitamin D, itself, is tightly bound by vitamin D binding protein and is the most highly lipid soluble form of vitamin D. For these reasons, 1,25(OH)2 vitamin D and vitamin D are not good indicators of a patient’s vitamin D status.

25(OH) vitamin D is a better indicator of the patient’s vitamin D status than the vitamin itself. This is because the hydroxy group makes 25(OH) vitamin D less fat soluble and makes it have a lower affinity to the vitamin D binding protein than the actual vitamin. These factors make the circulating concentrations of 25(OH) vitamin D about 1,000 times more concentrated than the steroid hormone form of vitamin D. 25(OH) vitamin D levels also correlate well with the clinical signs of vitamin D deficiency.

Vitamin D Reference Values
Although there is no consensus on 25(OH) vitamin D reference ranges, many leading authorities believe that health-based reference values are preferable.

Total Vitamin D Measurement
Physiological insufficiency is defined by the 25(OH) vitamin D concentrations below which parathyroid hormone (PTH) levels increase. When 25(OH) vitamin D concentrations are >30 ng/mL, PTH concentration levels off at its nadir and bone remodeling occurs at a normal rate. These sufficient levels of 25(OH) vitamin D and PTH are indicative of a state of normal bone remodeling. If 25(OH) vitamin D concentrations are reduced to <30 ng/mL, the PTH concentration increases and signals the up regulation of 1,25(OH)2 vitamin D which, increases the transport of intestinal calcium into the bloodstream and ultimately to the bone. The slightest increase in PTH is very important because it causes enhanced bone turnover and accelerated bone loss. When 25(OH) vitamin D drops to deficient levels (<20 ng/mL), classic signs of various rickets and osteomalacia are observed.

Two Forms of Vitamin D
There is another complicating factor in measuring 25(OH) vitamin D. Vitamin D and all of the metabolites have two distinct molecular forms: the vitamin D3 and vitamin D2 forms. There is an additional methyl group on the D3 form molecules, pictured below.

Vitamin D2 (Ergocalciferol)
Vitamin D3 (Cholecalciferol)

Although there is only minor difference in molecular structure, the forms have been shown to have very different efficacy in supplementation and treatment of bone density.

In two independent studies, using different dosing, vitamin D2 and D3 supplementation was compared. When 4,000 IU of vitamin D2 or D3 was administered daily to healthy individuals, the increase in the serum levels of 25(OH) vitamin D was 70% more for the group receiving vitamin D3. In another study, a single dose of 50,000 IU of vitamin D2 or D3 was given, the group receiving vitamin D3, had a significant increase in total 25(OH) vitamin D, while the patients given vitamin D2, had a decrease in total 25(OH) vitamin D after three weeks. These studies and the fact that only vitamin D3 has been effective in preventing bone loss or fractures in clinical trials prompted Houghton and Vieth to conclude in an article that “vitamin D3 should not be regarded as a nutrient suitable for supplementation or fortification.” As a result of these publications, the supplement industry recently reformulated many supplements from vitamin D2 to vitamin D3. Unfortunately, higher dose supplements and prescriptions in the United States still use vitamin D2. This makes it imperative to measure both 25(OH) vitamin D2 and 25(OH) vitamin D3 to make up the total 25(OH) vitamin D result.
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Although there is only minor difference in molecular structure, the forms have been shown to have very different efficacy in supplementation and treatment of bone density. In two independent studies, using different dosing, vitamin D2 and D3 supplementation was compared. When 4,000 IU of vitamin D2 or D3 was administered daily to healthy individuals, the increase in the serum levels of 25(OH) vitamin D was 70% more for the group receiving vitamin D3.7 In another study, a single dose of 50,000 IU of vitamin D2 or D3 was given, the group receiving vitamin D3 had a significant increase in total 25(OH) vitamin D, while the patients given vitamin D2 had a decrease in total 25(OH) vitamin D after three weeks.7 These studies and the fact that only vitamin D3 has been effective in preventing bone loss or fractures in clinical trials prompted Houghton and Vieth8 to conclude in an article that “vitamin D2 should not be regarded as a nutrient suitable for supplementation or fortification.” As a result of these publications, the supplement industry recently reformulated many supplements from vitamin D2 to vitamin D3. Unfortunately, higher dose supplements and prescriptions in the United States still use vitamin D2. This makes it imperative to measure both 25(OH) vitamin D2 and 25(OH) vitamin D3 to make up the total 25(OH) vitamin D result.
Dramatic Increase in Vitamin D Testing

The recent dramatic increase in vitamin D testing is primarily due to two causes. First, there has been a marked increase in vitamin D deficiency in the U.S. and throughout the world. It is estimated that one seventh of the world’s population is vitamin D deficient, and a recent study shows that nearly 80% of the U.S. population is insufficient. Many of the insufficiencies in the developed countries are due to people limiting their exposure to the sun to reduce their risk of skin cancer. People living near the equator who are exposed to sunlight without protection from the sun will normally have sufficient levels of vitamin D. However, vitamin D deficiency is found in these regions when individuals limit their exposure to direct sun by using clothing or sunblock. Unfortunately, the amount of vitamin D consumed in diets will not compensate for the reduction of vitamin D caused by the use of clothing or sunblock. The second reason for the increase in vitamin D testing is due to its use as a general health marker and the link between vitamin D deficiency and non-bone-related disorders:

- A prospective study indicated that women on supplementation had a 40% lower risk of developing multiple sclerosis than those who were not on a supplement.
- NHANES III (16,818 participants) showed that participants with a higher vitamin D level of ≥32 ng/mL had a 72% lower risk of colorectal mortality than those with a level <20 ng/mL.
- A Finnish study (10,366 children) showed that infants who had received 2,000 IU/day of vitamin D2 their first year of life were 80% less likely to develop type 1 diabetes, while children who were deficient had an increased risk of 200%.

The Vitamin D External Quality Assessment Scheme (DEQAS) has observed this dramatic increase in the increased enrollment in proficiency testing. In 2004, there were 141 participating laboratories. In 2009, the number of participating laboratories exploded to 670 in 35 countries. This increase in participating laboratories is reflective of the increase of 25(OH) vitamin D testing.

Below are a few of the cited statistical links between vitamin D deficiency and non-bone-related disorders:

- A recent study showed that 25(OH) vitamin D results differed widely depending on the laboratory and the method used, with the mean result (from 10 healthy adults) varying from 17.1 to 26.6 ng/mL. These authors found that a commercial chemiluminescent assay (CLIA) had the highest positive bias from the HPLC that was selected as their standard method. The July 2005 DEQAS proficiency survey included samples that had been spiked with 25(OH) vitamin D2 and 25(OH) vitamin D3. The two CLIA methods tested recovered 89% (D2), 81% (D3), and 56% (D2), 79% (D3), respectively, with both of these assays falling far below their reported reactivities: of 100% (D2), 100% (D3), and 70% (D2), 100% (D3) respectively. Both of these manufacturers have made corrections to their assays; however, in a recent publication, DEQAS shows that the first assay still has a negative bias of more than 7.0%, while the second assay has a mean bias of > +5.0%.

Wallace et al. also notes that the desired analytical sensitivity should be <10.0 ng/mL for 25(OH) vitamin D to detect severe deficiency. Its specificity needs to exclude significant interferences from the C-3 epimer of 25(OH) vitamin D, which is more prevalent in infants under one year old. These authors also point out that standardization needs to occur so 25(OH) vitamin D results can be more easily used by clinicians. Two key developments for standardizing 25(OH) vitamin D results are the availability of serum calibration standards that are standardized against the well-established reference materials, in addition to the development of reference methods. Recognizing the importance of a 25(OH) vitamin D2 and 25(OH) vitamin D3, reference material, the National Institute of Standards and Technology (NIST) recently released a four level Standard Reference Material set, SRM 972. Unfortunately, only one level of the new Standard Reference Material set has proven to be functional when used to standardize immunosassays. The other levels use horse serum or spike with exogeneous vitamin D. In initial testing using immunosassays, these levels have shown a significantly lower recovery, suggesting the possibility of matrix interferences. Additionally, the NIST has developed a candidate reference procedure using Isotope-Dilution LC/MS/MS. This method is too costly and labor intensive to be used routinely; however, it is valuable for standardization and companion purposes. Continued standardization efforts like these programs will enable laboratories to report more accurate and comparable 25(OH) vitamin D results, enabling better determination of a patient’s true vitamin D status.

Measuring Total Vitamin D

25(OH) vitamin D can be measured separately or as a total value, but not all immunosassays have the same reactivity to 25(OH) vitamin D2 and 25(OH) vitamin D3. Some immunosassays only detect one type of 25(OH) vitamin D and others do not fully detect the entire amount of each form of 25(OH) vitamin D. No matter which methodology you use, the most important use is the total serum 25(OH) vitamin D value because it represents the total amount of vitamin D (both D2 and D3) that is circulating, and it’s the same measure as used in the health-based reference values. This ensures that your patients receive the most reliable result to determine vitamin D status, regardless of level and type of supplementation.

To get a true reading of the patient’s vitamin D level, one has to use an immunosassay that detects both 25(OH) vitamin D2 and D3 equally. If the assay does not detect vitamin D2 fully or even partially, it is likely that the patient’s result will be reported in the insufficient range when the actual circulating concentration is sufficient. At the opposite end of the spectrum, when serum 25(OH) vitamin D levels are consistently >150 ng/mL (375 nmol/L), it is potentially toxic. Typically occurs due to vitamin D over-supplementation and is observed in patients taking more than the prescribed 40,000 IU per day. Toxicity due to sunlight overexposure and/or diet is unlikely. When vitamin D levels are this high, calcium concentrations rise as well and can result in nausea, weight loss, and constipation. As a result of increased levels of vitamin D and calcium, the patient can develop kidney stones. It is likely that supplementation at this level is vitamin D2; therefore, it is important that the assay has the same reactivity to 25(OH) vitamin D2 and 25(OH) vitamin D3.
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The second reason for the increase in vitamin D testing is due to its use as a general health marker and the link between vitamin D deficiency and several diseases. The diseases that have been statistically linked to vitamin D deficiency are various cancers, diabetes, multiple sclerosis, and cardiovascular and autoimmune diseases.

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Standardizing 25(OH) Vitamin D Assays

Although there has been a huge increase in 25(OH) vitamin D testing, much more needs to be done to truly standardize results. Brinkley et al.14 showed that 25(OH) vitamin D results differer widely depending on the laboratory and the method used, with the mean result from 10 healthy adults ranging from 17.1 to 35.6 ng/mL. These authors found that a commercial chemiluminescent assay (CLIA) had the highest positive bias from the HPLC that was selected as their standard method. The July 2005 DEQAS proficiency survey included samples that had been spiked with 25(OH) vitamin D2 and 25(OH) vitamin D3. The two CLIA methods tested recovered 89% (D2), 81% (D3) and 56% (D2), 79% (D3), respectively, with both of these assays falling far below their reported reactivities: of 100% (D2), 100% (D3), and 70% (D2), 100% (D3) respectively.15 Both of these manufacturers have made corrections to their assays; however, in a recent publication, DEQAS shows that the first assay still has a negative bias of more than 7.0%, while the second assay has a mean bias of +5.0%.16

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Recommendations for Clinical Practice

Recently, 25 experts from various medical disciplines drafted recommendations for vitamin D:

1. Use an assay that measures both 25(OH) vitamin D2 and 25(OH) vitamin D3.
2. Serum is the recommended sample type.
3. Report the total vitamin D results in ng/mL.
4. Participate in external quality control schemes that use human sera (e.g. DEQAS).
5. Internal quality control should use different levels. The College of American Pathologists recommends at least two levels.
6. Reports should include recommended total 25(OH) vitamin D health-based reference values, not population-based reference ranges. Reporting both ranges is confusing to the clinicians.

Reproducibility

In a 2009 study, reproducibility of commercially available assays in Australia and Canada were evaluated within lab and between labs to determine reproducibility in eight blinded participating clinical labs. The study also looked at the different precision levels at lower and higher end concentrations. The authors found significant imprecision issues not only within lab but between labs. According to the study, replicate testing in the same lab produced as much as a 97% variance; over half of the labs had replicates fluctuating at the clinical cut-off point.

"Overall, 40% of the 102 participants had replicate samples which differed by 20% or more, 29% of participants had results which differed by 30% or more, and 15% of participants had results which differed by more than 50% and 13% differed by more than 60%." Additionally, 13% of the subjects had contradicting results between laboratories, meaning they were considered insufficient in one lab but sufficient in another. Because of the unsatisfactory precision demonstrated by these assays, the author warns others about using these assays when making clinical decisions or for epidemiological findings.

Conclusion

Vitamin D testing likely continues to grow as studies link deficient vitamin D levels with more health conditions. Given the complexity of vitamin D results due to seasonality, it is important that laboratories deliver accurate and reliable results. A vitamin D assay that fully measures total 25(OH) vitamin D, is standardized, and has acceptable precision will allow the clinical lab to distinguish between patients who have deficiency, insufficiency, and sufficiency.

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References

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