Interpreting Vitamin D Assay Results: Proceed with Caution

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Abstract
Vitamin D deficiency and insufficiency are common in patients with CKD. Association studies suggest that low 25-hydroxyvitamin D3 (25-OH-D3) is a harbinger of poor outcomes in these patients. Serum 25-OH-D represents the best biomarker of vitamin D status, but there is much debate surrounding the performance of some of the assay methods. Programs such as the Vitamin D Standardization Program and Vitamin D External Quality Assessment Scheme (DEQAS) in the United Kingdom have allowed us to assess the accuracy and reproducibility of the available methods. Liquid chromatography–tandem mass spectrometry–based methods for serum 25-OH-D measurement are emerging as more accurate and reliable alternatives to the immunoassay-based methods that have dominated over the past 2 decades. There is a renewed optimism that serum 25-OH-D is a useful biomarker to be used in patients with CKD, in particular to diagnose vitamin D deficiency and monitor vitamin D therapy. This commentary discusses some of the methodologic problems associated with serum 25-OH-D assays and their resolution, and looks forward to the future of vitamin D testing.

Vitamin D Deficiency Is Common in Patients with CKD
It is now well established that vitamin D deficiency, not only the loss of the active form, 1,25-dihydroxyvitamin D3 (1,25-[OH]2D3; calcitriol), but also its immediate precursor, 25-hydroxyvitamin D3 (25-OH-D3), represents an important biomarker in patients with CKD (1,2). The evidence suggests that if left untreated, this results in secondary hyperparathyroidism and renal osteodystrophy, now identified as CKD–mineral and bone disorder. Kidney Disease Outcomes Quality Initiative guidelines recommend treating the deficiency of 25-OH-D3 with vitamin D supplements as well as treatment of the deficiency of 1,25-(OH)2D3 with calcitriol analogues (calcitriol, paricalcitol, doxercalciferol) starting at CKD stage 3 and escalating to use of both types of drugs by CKD stage 5 and during dialysis (3). Kidney Disease Improving Global Outcomes guidelines suggest that vitamin D deficiency and insufficiency in patients with CKD stages 3–5D be corrected using treatment strategies recommended for the general population but with a 2C weak grade of recommendation based on low quality of evidence (4). There is a consensus that serum total 25-OH-D (25-OH-D2 + 25-OH-D3) is a good biomarker for vitamin D status (5), and a wealth of evidence suggests that low total 25-OH-D correlates with many deleterious health outcomes, including indicators of bone fractures, cardiovascular health, immunologic health, musculoskeletal strength, and total mortality. Various studies have provided data to support that vitamin D3 therapy can correct low serum 25-OH-D, and in some cases the low 1,25-(OH)2D3 levels, in patients with CKD stages 3 and 4 (6,7). However, no clinical trial data suggest that correcting vitamin D deficiency improves health outcomes in CKD or ESRD. Late in CKD, calcitriol analogues represent essential tools in the suppression of secondary hyperparathyroidism.

Is It Necessary to Routinely Monitor Serum 25-OH-D as a Measure of Vitamin D Status?
The fall in serum 25-OH-D during the stages of CKD has been reported widely, and the finding of a low serum 25-OH-D is almost universal in dialysis units across North America. Is it therefore necessary to monitor serum 25-OH-D levels if it will be found to be low in most cases? The question is best answered by first defining the normal range for serum 25-OH-D. The Institute of Medicine (IOM) defined the deficiency/insufficiency threshold for serum 25-OH-D as <20 ng/ml (50 nmol/L) in the general population, even though Ca2+ and P(0.4)-related symptoms of vitamin D deficiency occur in most patients only when 25-OH-D levels fall below 12 ng/ml (30 nmol/L) (8). Because northerly latitudes (<30°N) do not permit vitamin D synthesis from October to April, serum 25-OH-D shows a seasonal variation, with highest levels in September/October and lowest in February/March. Consequently, IOM based its dietary reference intakes on the winter vitamin D requirements needed to maintain serum 25-OH-D <20 ng/ml (8). The Endocrine Society made recommendations for patients with disorders of calcium and phosphate homeostasis by describing vitamin D insufficiency as levels <30 ng/ml (75 nmol/L) (9,10).

Depending on which set of guidelines you accept, then, the different threshold makes the risk of deficiency greater or less. In fact, part of the recent explosion in
vitamin D testing in the general population has been due to this inflation of what is considered an adequate 25-OH-D to prevent vitamin D deficiency and insufficiency. But irrespective of the use of 25-OH-D assays for the general population, a stronger case can be made to routinely monitor serum 25-OH-D in patients with CKD. The problem is even more acute in the winter months, when there is no skin synthesis of vitamin D. Prophylactic vitamin D supplementation (both prescription vitamin D2 and over-the-counter vitamin D3 food supplements) has become popular in the treatment of patients with CKD. However, in the opinion of this vitamin D expert, levels still need to be monitored because of concerns about selecting the optimal loading and maintenance vitamin D dose; because of differences in patient’s ability to absorb, use, and adequately activate vitamin D into 25-OH-D; and because of concerns about vitamin D toxicity (11,12). Increasing body mass index correlates with a lower serum 25-OH-D and is presumed to be due in part to sequestration of the vitamin D in fat tissue (8,11). Consequently, some patients with CKD who have a higher body mass index may require higher vitamin D3 doses to normalize their serum 25-OH-D. Concerns about potential vitamin D toxicity are not so much related to acute toxicity—which we believe occurs only at serum 25-OH-D levels >150 ng/ml (375 nmol/L) (1,8,12)—but rather to long-term administration causing sustained 25-OH-D levels >50 ng/ml (125 nmol/L) (8). Recent data suggest that optimal serum 25-OH-D levels range between 20–50 ng/ml and follow a U-shaped or reverse J-shaped curve with such end points as total mortality or cancer rates increasing at either end of this range (13–15). Thus, serum 25-OH-D should be monitored to make sure patients are in the optimal range; low serum 25-OH-D should be corrected and serum 25-OH-D should continue to be checked about every 6 months or yearly to ensure that the patient responds adequately.

Are All Current Serum 25-OH-D Assays Accurate?

I wish that I could say yes, but the truth is that although assays continue to improve with time, the average coefficient of variation is still around 10%–15% and the bias (negative or positive) of some assays is as high as 20%–30% (16). Hence the title of this review: proceed with caution. Serum 25-OH-D assays fall into two main categories: (1) those based on a separation step of chromatography, the most popular of which is liquid chromatography–tandem mass spectrometry (LC-MS/MS) and (2) nonchromatographic antibody-based methods based on antibody or protein binding, such as radioimmunoassays. The LC-MS/MS methods are rapidly gaining in popularity because they are inherently more consistent (coefficient of variation <5%–10%) and much more accurate (bias <5%) than antibody-based methods. Three “policing” bodies—the Vitamin D Standardization Program (initiated by the US Office of Dietary Supplements in the National Institutes of Health in collaboration with the National Institute of Standards and Technology, Centers for Disease Control and Prevention, and Ghent University) (17,18), Vitamin D External Quality Assessment Scheme (DEQAS) based in London, United Kingdom (19), and the College of Pathologists—now monitor performance and accredit laboratories engaged in serum 25-OH-D assays.

DEQAS circulates 20 serum samples per year and has >1200 participants. Because it requires identification of assay methods used, the scheme allows a look at both individual laboratory performance and trends in the different methods. DEQAS testing has identified several methodologic problems over the past decade, leading to the withdrawal or redesign of commercial antibody-based methods (16,19). Problems include the poor performance of such methods for sera containing 25-OH-D2 or overestimation of total 25-OH-D by the inclusion of the catabolite, 24,25-(OH)2D3 (20,21). In general, LC-MS/MS performs better in these external assessment schemes than all other methods. However, even LC-MS/MS is not immune from technical issues associated with detection and overestimation of 25-OH-D by the inclusion of 3-epi-25-OH-D3 in test results. This minor metabolite of unknown biologic importance is found in highest concentration during the neonatal period and represents about 6% of total 25-OH-D in adults (22–24). Reported total serum 25-OH-D values should include 25-OH-D3 and 25-OH-D2 but not 3-epi-25-OH-D3. Analysts can easily resolve the 3- epoxy-25-OH-D3 from 25-OH-D3 using current liquid chromatography columns; however, on the basis of DEQAS results, there is a suspicion that some LC-MS/MS laboratories still choose to ignore the 3-epi-25-OH-D3 issue and sacrifice some accuracy for increased throughput (16). Others claim that in adults the difference between the results of LC-MS/MS methods that resolve 3-epi-25-OH-D3 and those that do not are so small as to be explained by analytical imprecision rather than the presence of 3-epi-25-OH-D3 interference (25).

Recent data suggest that automated versions of nonchromatographic antibody-based assays, which are often used in clinical chemistry laboratories for higher throughput, have particular problems with a step involving the release of the analyte 25-OH-D, from the vitamin D–binding protein (DBP) (26). Is this a surprise? Not really, since the same issues plague testosterone release and measurement. At least one major journal, the Journal of Clinical Endocrinology and Metabolism, from January 1, 2015, onward will require that research articles in which sex steroid assays are important endpoints be based on mass spectrometry; the editors will no longer accept immunoassay methods for this purpose (26). One can anticipate that for vitamin D analysis, LC-MS/MS-based methods will become the chosen approach in clinical research in the future.

Do Routine Serum 25-OH-D Assays Need to Be Accurate?

LC-MS/MS instruments are expensive and may be inferior to automated analyzers in terms of sample throughput. Hospitals have financial and logistic reasons for continuing the less accurate immunoassay methods for routine patient assessment. An argument put forward to counter the notion that serum 25-OH-D must involve LC-MS/MS technology is that it is too expensive for routine use and its preoccupation with precision and accuracy is overkill and less important than knowing the general vitamin D status of a given patient: deficiency, sufficiency, or toxicity. Binkley points out that knowing the method-related thresholds and method variability may be more relevant than using fixed IOM values for vitamin deficiency based on LC-MS/MS technology (27,28).
By this approach, the physician becomes familiar with the vitamin D thresholds for the chosen assay rather than the IOM-specified threshold value (>20 ng/ml) required to ensure vitamin D sufficiency. However, a caution must be sounded that although this approach may work to follow responses of individual patients before and after vitamin D therapy, the imprecise methods should not be used in research studies involving patient-to-patient or method-to-method comparisons. All methods used for research or routine purposes should be subject to external quality assessment schemes (DEQAS, Vitamin D Standardization Program, and College of American Pathologists), which in the main-use samples with National Institute of Standards and Technology reference values, in the process providing more confidence that the assays will meet current accuracy standards. The Vitamin D Standardization Program is using its program to coordinate efforts to bring all LC-MS/MS methods and immunoassay manufacturers into line based upon different performance criteria (17).

**Future Directions**

There has been much debate recently around the importance of bioavailable 25-OH-D3 since there are claims (29) that it may explain the paradox that serum 25-OH-D levels are lower in African Americans than in light-skinned population groups (30), yet the risk for fracture is lower. Levels are lower in African Americans than in light-skinned (29) that it may explain the paradox that serum 25-OH-D levels are lower in African Americans than in other ethnic groups (31). 25-OH-D is a lipid-soluble molecule that circulates in the bloodstream mainly bound to an ε-globulin, vitamin D-binding protein. 25-OH-D is postulated to enter cells in a “free form” similar to other steroid hormones so that the “bioavailable 25-OH-D3” (defined as the fraction of total 25-OH-D that is unbound to any protein or bound to albumin and not that fraction bound to vitamin D-binding globulin [DBP]). It is believed that bioavailable 25-OH-D3 may be a more useful parameter than total 25-OH-D or DBP-bound 25-OH-D that better correlates with the health-promoting effects of vitamin D. The story is further complicated because DBP exists in three main isoforms (Gc1F, Gc2, and Gc1S), each with different affinities for 25-OH-D3, and these isoforms segregate to different ethnic groups. The total concentration of DBP varies over a fairly wide range. African Americans who regularly have very low total 25-OH-D levels in the face of normal bone health (26,29) exhibit the high-affinity Gc1F isoform and lower total DBP concentrations than white Americans, who generally have the Gc1S isoform. Overall, Powe et al. (29) propose that bioavailable 25-OH-D is similar in both groups despite large differences in total 25-OH-D levels (26). However, others (32) have produced data to refute Powe and colleagues’ claims on the basis of the fact that antibodies used by Powe et al. do not detect all isoforms of DBP equally. Consequently, it appears that methods required to measure bioavailable 25-OH-D are still in their infancy and have the same problems as other antibody-based methods. Nevertheless, this parameter might gain popularity if it is shown to be a better correlate with clinical endpoints than total 25-OH-D.

As noted earlier, the accuracy and lower variability of LC-MS/MS technology have revolutionized the analysis of many small bioactive molecules, some of which are relevant to the nephrologist. The broad versatility of LC-MS/MS technology allows for the simultaneous measurement of several analytes in a single run, so that it has the advantage of providing multiple analytes at the same time. Some LC-MS/MS laboratories are developing methods that simultaneously measure all clinically relevant vitamin D metabolites: 25-OH-D3, the active form, 1,25-(OH)2D3, and the catabolite, 24,25-(OH)2D3, using 100–250 μl of serum. These resulting vitamin D metabolic profiles permit the calculation of ratios of different metabolites. The usefulness of this approach is illustrated in idiopathic infantile hypercalcemia (33), where loss-of-function mutations of CYP24A1 lead to very low 24,25-(OH)2D3 levels and elevations of 25-OH-D3 and 1,25-(OH)2D3 (34). The clinical consequence is hypercalcemia and hypercalcemia, which, over time, result in nephrocalcinosis and nephrocalcinosis. The ratio of 25-OH-D3 to 24,25-(OH)2D3 rises from approximately 10:1 in healthy persons to approximately 100:1 in patients with idiopathic infantile hypercalcemia. So the demand by the clinician for multiple vitamin D metabolites to aid in diagnosis of rare calcium- and phosphate-related diseases is likely to be satisfied by improvements in LC-MS/MS technology.

One United States pharmaceutical company is in phase III clinical trials with a slow-release form of 25-OH-D3 to be used for treating secondary hyperparathyroidism in patients with stages 3, 4, and 5 CKD. This agent, which is still used in Europe and was available in the United States as Calderol from 1970 to 2000, raises serum 25-OH-D more predictably and more quickly than vitamin D itself (35). Patients treated with this agent will need to be monitored using serum 25-OH-D measurements to assess whether they are restored to the 20–50 ng/ml range recommended by the IOM.

Accordingly, although the title of this commentary has a cautious tone, there is much in this field to be optimistic about. The assay technology is being constantly monitored; the emergence of LC-MS/MS to measure 25-OH-D more accurately continues, while at the same time offering the bonus of other potentially important vitamin D metabolites; and the serum 25-OH-D3 value is a useful parameter with which the nephrologist can assess disease and the risk of bone disease in CKD–mineral and bone disorder and monitor current and future therapies.

**Disclosures**

G.J. was a member of the Institute of Medicine committee that reviewed Dietary Reference Intakes for Calcium and Vitamin D, 2011; he is a consultant and Science Advisory Board member of OPKO Renal, which is developing vitamin D drugs for the treatment of secondary hyperparathyroidism; he is a member of the Advisory Panel of DEQAS; he is a contributing investigator and is funded by the Vitamin D Standardization Program (Office of Dietary Supplements and National Institute of Standardization Technology); and he is funded by the European Rare Diseases and Canadian Institutes for Health Research to study idiopathic infantile hypercalcemia.

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