Measuring 25-hydroxyvitamin D in a clinical environment: challenges and needs

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ABSTRACT
The number of assessments of circulating 25-hydroxyvitamin D [25(OH)D] for diagnostic purposes has increased significantly during the past 5 y. Over the years, techniques for quantifying 25(OH)D have increased and evolved. Some changes have been for the better and some have not. Most current methods appear to provide valid results as long as the operator of the given technique is properly trained and motivated. In addition, any laboratory that assesses circulating 25(OH)D for clinical diagnosis needs to participate in the Vitamin D External Quality Assessment Scheme. Finally, research has shown that circulating 25(OH)D is extremely stable in stored serum or plasma samples; this characteristic makes accurate, long-term epidemiologic studies of circulating 25(OH)D possible. In this article, I provide an overview of the techniques available for measuring 25(OH)D, compare these techniques with one another, and assess their clinical utility. I also briefly discuss the stability of 25(OH)D in biological media and present an overview of the Vitamin D External Quality Assessment Scheme. 

INTRODUCTION
Vitamin D is a 9,10-seco steroid, as shown by the numbering of its carbon skeleton. Vitamin D has 2 distinct forms: vitamin D<sub>2</sub> and vitamin D<sub>3</sub>. Vitamin D<sub>2</sub> is a 28-carbon molecule derived from the plant sterol ergosterol, whereas vitamin D<sub>3</sub> is a 27-carbon derivative of cholesterol. Vitamin D<sub>2</sub> differs from vitamin D<sub>3</sub> in that it contains an extra methyl group and a double bond between carbons 22 and 23.

The most important aspects of vitamin D chemistry center on its cis-triene structure. This unique structure makes vitamin D and related metabolites susceptible to oxidation, ultraviolet (UV) light-induced conformational changes, heat-induced conformational changes, and attacks by free radicals. Most of these transformation products have less biological activity than does vitamin D. Research has now shown that vitamin D<sub>3</sub> is much less bioactive than vitamin D<sub>2</sub> in humans (1). The parent compounds vitamin D<sub>2</sub> and vitamin D<sub>3</sub> are sometimes referred to as calciferol.

Hydroxylation reactions at both carbon 25 of the side chain and, subsequently, carbon 1 of the A ring result in the metabolic activation of vitamin D. Metabolic inactivation of vitamin D takes place primarily through a series of oxidative reactions at carbons 23, 24, and 26 of the molecule’s side chain. Metabolic activation and inactivation are well characterized and result in a plethora of vitamin D metabolites (2). Of these metabolites, only 25-hydroxyvitamin D [25(OH)D] and 1,25-dihydroxyvitamin D [1,25(OH)<sub>2</sub>D] provide any clinically relevant information. 25(OH)D<sub>2</sub> and 25(OH)D<sub>3</sub> are commonly known as calcifediol and the 1,25(OH)<sub>2</sub>D metabolites as calcitriol.

Here I describe the current state of the science on the clinical assessment of circulating 25(OH)D. Information on 1,25(OH)<sub>2</sub>D assessment is available elsewhere (3).

DETECTION OF 25(OH)D: COMPETITIVE PROTEIN BINDING ASSAY
A major factor responsible for the explosion of information on vitamin D metabolism and its relation to clinical disease was the introduction of a competitive protein binding assay (CPBA) for 25(OH)D. John Haddad, Jr, introduced this CPBA >3 decades ago. The assay assessed circulating 25(OH)D concentrations by using the vitamin D binding protein (DBP) as a primary binding agent and <sup>3</sup>H-25(OH)D<sub>3</sub> as a reporter (4). Although this CPBA was valid, it was also relatively cumbersome. Technicians had to extract the sample with organic solvent, dry it under nitrogen, and purify it by using column chromatography. In addition, the assay required individual sample recovery estimates to account for endogenous losses of 25(OH)D during this extensive procedure. This assay was suitable for the research laboratory but did not meet the requirements of a high-throughput clinical laboratory. As a result, the quest began to simplify the method used to assess circulating 25(OH)D.

The major difficulty in measuring 25(OH)D is attributable to the molecule itself. 25(OH)D is probably the most hydrophobic compound measured by a protein binding assay (PBA), which constitutes CPBA or radioimmunoassay (RIA). The fact that the molecule exists in 2 forms, 25(OH)D<sub>2</sub> and 25(OH)D<sub>3</sub>, compounds the difficulties associated with its aqueous insolubility. 25(OH)D’s lipophilic nature renders it especially vulnerable to the matrix effects of any PBA. Something present in the sample assay tubes that is not present in the standard assay tubes can cause matrix effects. These matrix effect substances are usually lipids, but in the newer direct assays, they could be anything contained in the serum or plasma sample. The matrix factors...
change the ability of the binding agent, antibody, or binding protein to associate with 25(OH)D in the sample or standard in an equal fashion. When this occurs, it markedly diminishes the assay’s validity. Experience has shown that the DBP is more susceptible to these matrix effects than are antibodies (5). The original Haddad procedure overcame the matrix problem by using chromatographic sample purification before CPBA (4).

Researchers had a strong desire to simplify the CPBA for circulating 25(OH)D, so Belsey et al (6) developed a new CBPA in 1974. The goal of this second-generation CPBA was to eliminate chromatographic sample purification as well as individual sample recovery by using 1H-25(OH)D3. However, researchers were unable to validate the Belsey assay because of matrix problems originating from ethanoic sample extraction.

In 1978, after several years of trying to validate nonchromatographic CPBA measurements of circulating 25(OH)D, researchers abandoned the method. Dorantes et al (7) showed that unknown substances in unchromatographed samples interacted with the DBP to cause spurious results in the CPBA; this finding sealed the fate of nonchromatographic CPBA for 25(OH)D. As a result, investigators and clinicians had to return to the Haddad method (4) or the newly introduced direct UV HPLC assay, which are slow, cumbersome, and expensive (8). However, the early 25(OH)D CPBA of Belsey and colleagues (6) did have the advantage of being co-specific for 25(OH)D2 and 25(OH)D3 and thus provided a total 25(OH)D value.

This DBP’s binding co-specificity for 25(OH)D2 and 25(OH)D3, as well as its stability, made it an attractive candidate for incorporation into automated direct chemiluminescent assays. In fact, the Nichols Institute used this approach when its researchers developed the Advantage 25-hydroxyvitamin D assay. The US Food and Drug Administration approved this assay for clinical use, but Nichols Institute Diagnostics ultimately withdrew it from the marketplace due to its propensity to overestimate total circulating 25(OH)D concentrations and its inability to detect circulating 25(OH)D2 (9). Although never described, these problems were probably linked to the DBP’s inability to resolve the matrix problems associated with a direct sample assay. Currently, the CPBA for 25(OH)D is rarely used. One cannot accurately compare CPBA results for circulating 25(OH)D concentrations from the past with values from current methods because many of the matrix interferences were not linear in the old CPBAs.

**RADIOIMMUNOASSAY**

In the early 1980s, my group decided that a nonchromatographic RIA for circulating 25(OH)D would be the best approach to measuring 25(OH)D. We therefore designed an antigen that would generate an antibody that was co-specific for 25(OH)D2 and 25(OH)D3 (10). In addition, we designed a simple extraction method that allowed simple nonchromatographic quantification of circulating 25(OH)D (10). We further modified this assay in 1992 to incorporate a 125I-labeled reporter and calibrators (standards) in a serum matrix. This finally made mass assessment of circulating 25(OH)D possible (11).

My group’s DiaSorin RIA for 25(OH)D (DiaSorin Corporation, Stillwater, MN) has set the standard for clinical diagnosis of nutritional vitamin D deficiency. Investigators have also used it to conduct virtually all of the research linking circulating 25(OH)D to various diseases. It is still in widespread use in 2007 because it is the only RIA that detects total circulating 25(OH)D.

**DIRECT DETECTION METHODS**

Direct detection methods for determining circulating 25(OH)D include both HPLC and liquid chromatography-mass spectrometry (LC-MS) procedures (8, 12). The HPLC methods separate and quantitate circulating 25(OH)D2 and 25(OH)D3 individually. HPLC followed by UV detection is highly repeatable and, in general, most persons consider it the gold standard method. However, these methods are cumbersome and require a relatively large sample as well as a radioactive internal standard. Sample throughput is slow, and a highly trained, dedicated technician needs to perform this method accurately. Thus, HPLC is not suited to a high-capacity clinical laboratory.

Researchers have recently revitalized LC-MS as a viable method for assessing circulating 25(OH)D (13). As with HPLC, LC-MS quantitates 25(OH)D2 and 25(OH)D3 separately. When performed properly, LC-MS is an accurate testing method. However, the equipment is very expensive, and its overall sample throughput cannot match that of the automated instrumentation format. As a method, LC-MS compares favorably with RIA techniques (13).

One unique problem with LC-MS is its relative inability to discriminate between 25(OH)D2 and its inactive isomer 3-epi-25(OH)D3. This problem has been especially noticeable in the circulation of newborn infants (13). As with HPLC, LC-MS requires an internal standard for each compound quantitated and a highly trained technologist to operate the system. However, its sample throughput can be much higher than that of HPLC.

**RANDOM-ACCESS AUTOMATED INSTRUMENTATION**

DiaSorin Corporation, Roche Diagnostics (Indianapolis, IN), and the now-defunct Nichols Institute Diagnostics all introduced methods for the direct (no extraction) quantitative determination of 25(OH)D in serum or plasma by using competitive chemiluminescence technology (9, 14). These assays appear quite similar on the surface but they are not.

In 2001, Nichols Diagnostics introduced the fully automated chemiluminescence Advantage 25(OH)D Assay System. In this assay system, unextracted serum or plasma is added directly into a mixture containing human DBP, acridinium-ester-labeled anti-DPB, and 25(OH)D3-coated magnetic particles. Note that the primary binding agent was human DBP. Thus, this assay was a CPBA, much like the manual procedure introduced in 1974 by Belsey et al (6). The major difference between these procedures was that Belsey deproteinized the sample with ethanol before assaying it. The calibrators for the Belsey assay were in ethanol. In the Advantage assay, the calibrators were in a serum-based matrix, and its developers assumed that this matrix would replicate the serum or plasma sample introduced directly into the assay system. In the end, the 1974 Belsey assay never worked and neither did the Advantage 25(OH)D Assay. As mentioned earlier, the Advantage assay constantly overestimated total 25(OH)D levels and was unable to detect 25(OH)D2 in patients treated with vitamin D3 (11). The company took the assay out of the marketplace in 2006.

In 2004, the DiaSorin Corporation introduced the fully automated chemiluminescence Liaison 25(OH)D Assay System (14). This assay was very similar to the Advantage assay, with one major difference: the Liaison assay used an antibody as a primary binding agent as opposed to human DBP in the Advantage system. Thus, the Liaison is a true RIA method. Details on...
this procedure are available elsewhere (14). The Liaison 25(OH)D assay is co-specific for 25(OH)D$_2$ and 25(OH)D$_3$, so it reports a total 25(OH)D concentration. Furthermore, it has gained wide acceptance and is used in many large clinical reference laboratories throughout the world. Recently, DiaSorin introduced a second-generation Liaison 25(OH)D assay, and the US Food and Drug Administration approved it for clinical use. This new version has increased functional sensitivity and has much improved assay precision.

The most recent addition to the automated 25(OH)D assay platforms is from Roche Diagnostics. Their test is an RIA called Vitamin D$_3$ (25-OH) and it can be performed on their Elecsys and Cobas systems. Roche released this assay in 2007, so little information on it is available. However, the assay can detect only 25(OH)D$_3$, so it will not be a viable product in countries in which vitamin D$_2$ is used clinically, including the United States.

CHOOSING AN ASSAY PLATFORM

Clinicians and researchers must consider several factors when choosing a platform. If they do not plan to perform the assay frequently, they should consider developing an in-house assay that uses HPLC rather than purchasing commercial kits, because commercial kits produce more variable results when inexperienced technicians use them (15). In addition, HPLC methods allow the technician to monitor the performance of standards in real time and to develop recovery estimates by using $^3$H-25(OH)D$_3$; these features make it easier to address problems when they arise.

For laboratories requiring higher throughput, a commercially available manual RIA is more appropriate because of its higher throughput capacity. The highest throughput reference laboratories typically use automated platforms or automated LC-MS because they can process thousands of samples per day with these methods.

When considering commercially available assays, one must ensure that the assay can recognize both vitamin D$_2$ and vitamin D$_3$. All of the commercial assays, except for the DiaSorin products, tend to underestimate 25(OH)D concentrations in patients treated with vitamin D$_2$. The use of vitamin D$_2$ supplements is declining. However, the sole therapeutic form of vitamin D in the United States is Drisdol (ergocalciferol, or vitamin D$_2$, made by United States). Drisdol has been used in many countries in which vitamin D$_2$ is used clinically, including the United States.

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CLINICAL REPORTING OF CIRCULATING 25(OH)D CONCENTRATIONS

As difficult as it is to believe, the clinical community has begun to debate whether it is better to report separate values for circulating 25(OH)D$_2$ and 25(OH)D$_3$ as opposed to a total 25(OH)D value. A total 25(OH)D value is simply 25(OH)D$_2$ and 25(OH)D$_3$ added together. The field has been determining 25(OH)D$_2$ and 25(OH)D$_3$ separately by HPLC for nearly 3 decades, adding them together, and providing a total 25(OH)D value to the clinician, which is what clinicians need for diagnosis.

Why then is the debate occurring? Simply, the debate is occurring because of the widespread use of LC-MS technology by clinical reference laboratories. As I mentioned earlier in this text, because of the nature of the LC-MS assay, laboratories that use this method must quantitate 25(OH)D$_2$ and 25(OH)D$_3$ separately. Some of these reference laboratories advocate reporting separate values because they claim that doing so is superior to simply adding them together to create a total 25(OH)D value. Absolutely no scientific evidence, not even a single publication, supports this claim, however. Some reference laboratories have actually charged separately for 25(OH)D$_2$ and 25(OH)D$_3$ measurements by charging inappropriate Current Procedural Terminology (CPT) codes. This practice is unethical and must not continue. The only CPT code for 25(OH)D (calcifiediol) reimbursement is 82306, which should be the only one billed. In fact, research has actually shown that this type of separate reporting confuses clinicians (16). The fact is that it is total 25(OH)D that actually drives a clinician’s decision to treat. Furthermore, 99% of all patients tested will have no circulating 25(OH)D$_2$ because 25(OH)D$_2$ will be present only if the patient is under treatment with Drisdol.

This is not to say that the separate determination of 25(OH)D$_2$ and 25(OH)D$_3$ is not useful in a research environment; it is. We have been using this separation in our research for nearly 3 decades (1, 17, 18). It simply has no special clinical diagnostic utility.

STABILITY OF 25(OH)D AND 1,25(OH)$_2$D IN SERUM OR PLASMA

Researchers have known for nearly 30 y that endogenous 25(OH)D and 1,25(OH)$_2$D are extremely stable in serum or plasma (19). Lissner et al (19) showed that vitamin D metabolites in blood stored at 24 °C for up to 72 h remain intact. Recent studies on the stability of 25(OH)D in plasma or serum that has undergone many freeze-thaw cycles have reported the same stability (14, 20). I have used the same pooled human 25(OH)D and 1,25(OH)$_2$D internal controls stored at −20 °C for >10 y with no detectable degradation of either compound. The Vitamin D External Quality Assessment Scheme (DEQAS), a major vitamin D quality assessment organization, ships its serum samples used by laboratories for quality assessment by ground post worldwide without affecting 25(OH)D and 1,25(OH)$_2$D values.

I have performed experiments to try to destroy endogenous 25(OH)D and 1,25(OH)$_2$D in plasma to obtain a vitamin D–free human plasma to prepare various immunoassay procedure calibrators. When I placed crystalline 25(OH)D$_2$ or 1,25(OH)$_2$D$_2$ in ethanol in an open glass petri dish and exposed the dish to intense UV light, the UV light destroyed the compounds within a few minutes. When I conducted the same experiment by using serum or plasma, however, the 25(OH)D$_2$ and 1,25(OH)$_2$D$_2$ levels did not change after 2 d of UV light exposure. I therefore stopped trying to use this procedure to produce vitamin D–free plasma.

Why are vitamin D and its metabolites so stable in serum or plasma when they are insulted with UV light, temperature shifts, or oxidation? One reason is that UV light penetrates aqueous media very poorly. However, the main reason is probably that in serum or plasma, vitamin D and its metabolites are essentially bound completely to the serum DBP and this complex resists potential insults to the vitamin D molecule very effectively. In conclusion, 25(OH)D and 1,25(OH)$_2$D are very stable in serum or plasma, so they require only minimal attention to storage conditions.
THE VITAMIN D EXTERNAL QUALITY ASSESSMENT SCHEME

DEQAS (Internet: www.deqas.org) was founded in 1989 to compare the performance of then-available 25(OH)D tests. DEQAS has since become the largest vitamin D quality assessment program in the world, with ≈400 participating laboratories worldwide. The organization’s major aim today is to assess the analytic reliability of 25(OH)D and 25(OH)2D assays. The organization achieves this goal by

- Distributing serum pools at regular intervals;
- Conducting statistical analyses of submitted results;
- Appropriately manipulating pools to provide information on assay specificity and recovery;
- Assigning GC-MS target values to selected 25(OH)D pools;
- Helping participants and manufacturers evaluate methods by providing samples, technical support, and impartial advice;
- Offering advice and support to participants having difficulty achieving an acceptable level of assay performance; and
- Providing a forum for exchanging information on all aspects of vitamin D assay methodology.

My laboratory has participated in both the DEQAS 25(OH)D and the DEQAS 1,25(OH)2D survey since 1997, and the survey has been invaluable in maintaining the integrity of our assay procedures. When DEQAS leaders question manufacturers about inconsistencies in their methods, most manufacturers attempt to address the issue identified.

One example of the value DEQAS offers occurred when DEQAS informed Nichols Institute Diagnostics that its Advantage 25(OH)D automated assay was overestimating total 25(OH)D concentrations and that, contrary to the manufacturer’s claims, the method could not detect circulating 25(OH)D2 concentrations (9). Nichols Institute Diagnostics chose not to respond to the concerns that DEQAS identified. The company subsequently went out of business and its Advantage 25(OH)D assay is no longer on the market. As this example shows, DEQAS provides an invaluable service to the vitamin D assay community. In the future, I hope that DEQAS can incorporate the new National Institute of Standards and Technology 25(OH)D calibrators into its survey in some fashion.

The DEQAS survey has shown that most current 25(OH)D assay protocols perform in a comparable fashion with respect to absolute values, assay linearity, and assay precision. However, the survey results also show that the only assays that quantitatively detect total 25(OH)D are HPLC methods, LC-MS methods, and the DiaSorin assays.

CONCLUSION

I have described the history of vitamin D assay development. The first 25(OH)D assays were largely nonchromatographic CBPAs that were invalid due to interactions between the DBP and substances in unchromatographed samples. When developers incorporated DBP into automated direct chemiluminescent assays, these assays tended to overestimate circulating 25(OH)D concentrations and could not detect circulating 25(OH)D2. The DiaSorin RIA uses an antibody that is co-specific for 25(OH)D2 and 25(OH)D3 and allows nonchromatographic quantification of total circulating 25(OH)D. Laboratories now use this assay for clinical diagnosis of vitamin D deficiency. Laboratories can also measure circulating 25(OH)D directly by using HPLC and LC-MS, but these more time-consuming assays require far more expensive equipment.

I have also discussed the importance of reporting total 25(OH)D concentrations to clinicians, who base their decisions on the total values rather than on separate 25(OH)D2 and 25(OH)D3 concentrations. I showed that vitamin D and its metabolites are stable in serum and plasma and therefore do not need elaborate storage arrangements. Finally, I discussed the value of the services provided by DEQAS to ensure the quality of vitamin D assays.

The author is an academic consultant to the DiaSorin Corporation (Stillwater, MN), the leading manufacturer worldwide of vitamin D assay devices approved by the Food and Drug Administration.

REFERENCES

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