

# Assessment and Interpretation of Circulating 25-Hydroxyvitamin D and 1,25-Dihydroxyvitamin D in the Clinical Environment

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## KEYWORDS

- Vitamin D • 25-Hydroxyvitamin D
- 1,25-Dihydroxyvitamin D • Calcidiol • Calcitriol

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 biologic activity than does vitamin D. Research has now shown that vitamin D<sub>2</sub> is much less bioactive than vitamin D<sub>3</sub> in humans.<sup>1</sup> 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.<sup>2</sup> Of

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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.

In this review the current state of the science on the clinical assessment of circulating 25(OH)D and 1,25(OH)<sub>2</sub>D is described.

## METHODS OF 25(OH)D QUANTITATION

The assessment of circulating 25(OH)D started its journey approximately 4 decades ago with the advent of the competitive protein-binding assay (CPBA).<sup>3</sup> From that early time to the present we have progressed to radioimmunoassay (RIA), high-performance liquid chromatography (HPLC), and liquid chromatography coupled with mass spectrometry (LC/MS). A brief description of each technique is given here.

### *Competitive Protein-Binding Assay*

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A major factor responsible for the explosion of information on vitamin D metabolism and its relation to clinical disease was the introduction of a CPBA for 25(OH)D. Haddad and Chyu<sup>3</sup> introduced this CPBA almost 4 decades ago. The assay assessed circulating 25(OH)D concentrations 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. 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 using column chromatography. This assay was suitable for the research laboratory but did not meet the requirements of a high-throughput clinical laboratory.

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 protein-binding assay (PBA), which constitutes either 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 with its quantitation by PBA. 25(OH)D's lipophilic nature renders it especially vulnerable to the matrix effects of any PBA. Anything present in the sample assay vessel that is not present in the calibrator assay vessel can cause matrix effects. These matrix effect substances are usually lipid but in the newer direct assays, they could be anything contained in the serum or plasma sample. These 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 demonstrated that the DBP is more susceptible to these matrix effects than antibodies.<sup>4</sup> The original Haddad procedure overcame the matrix problem by using chromatographic sample purification before CPBA.<sup>3</sup>

Researchers had a strong desire to simplify this cumbersome CPBA for 25(OH)D, so Belsey and colleagues<sup>5</sup> developed a streamlined CPBA in 1974. The goal of this second-generation CPBA was to eliminate chromatographic sample purification as well as individual sample recovery using <sup>3</sup>H-25(OH)D<sub>3</sub>. However, after several years of trying, researchers were unable to validate the Belsey assay due to matrix problems originating from ethanolic sample extraction.<sup>6</sup>

The 25(OH)D CPBAs did have the advantage of being cospecific for 25(OH)D<sub>2</sub> and 25(OH)D<sub>3</sub> and thus provided a "total" 25(OH)D value if the assay was valid. The DBP's binding cospecificity for 25(OH)D<sub>2</sub> and 25(OH)D<sub>3</sub> as well as its stability made it an attractive candidate for incorporation into automated direct chemiluminescent assays. In fact, Nichols Institute Diagnostics used this approach when its researchers developed the Advantage 25(OH)D Assay. The US Food and Drug Administration (FDA) approved this assay for clinical use, but Nichols ultimately withdrew it from

the market place due to its propensity to overestimate total circulating 25(OH)D concentrations and its surprising inability to detect circulating 25(OH)D<sub>2</sub>.<sup>7,8</sup> Although never described, these problems were probably linked to the DBP's inability to resolve the matrix problems associated with direct sample assay. At present, the CPBA for 25(OH)D is rarely used. Also, one cannot accurately compare most 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**

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In the early 1980s, the author's group decided that a nonchromatographic RIA for circulating 25(OH)D would be the best approach to measuring the substance. This group therefore designed an antigen that would generate an antibody that was cospecific for 25(OH)D<sub>2</sub> and 25(OH)D<sub>3</sub>.<sup>9</sup> In addition, a simple extraction method was designed that allowed simple nonchromatographic quantification of circulating 25(OH)D.<sup>9</sup> In 1985 Immunonuclear Corp., now known as DiaSorin, introduced this <sup>3</sup>H-based RIA as a kit on a commercial basis. This RIA was further modified in 1993 to incorporate a <sup>125</sup>I-labeled reporter and calibrators (standards) in a serum matrix.<sup>10</sup> This modification finally made mass assessment of circulating 25(OH)D possible. In that same year this assay became the first FDA-approved device for the clinical diagnosis of nutritional vitamin D deficiency. Further, during the past 23 years these DiaSorin tests have been used in the vast majority of large clinical studies worldwide to define "normal" circulating 25(OH)D levels in a variety of disease states. This test still remains today the only RIA-based assay that provides a "total" 25(OH)D value.

### **Random-Access Automated Instrumentation**

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DiaSorin Corporation, Roche Diagnostics, 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 using competitive protein assay chemiluminescence technology.<sup>11</sup> 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, nonextracted serum or plasma was added directly into a mixture containing human DBP, acridinium-ester labeled anti-DBP, and 25(OH)D<sub>3</sub>-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 and colleagues.<sup>5</sup> The major difference between these procedures was that Belsey depotenized 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. The company removed the assay from the market in 2006.

In 2004, the DiaSorin Corporation introduced the fully automated chemiluminescence Liaison 25(OH)D Assay System.<sup>11</sup> This assay is very similar to the late Advantage assay, with one major difference: the Liaison assay uses an antibody as a primary binding agent as opposed to the human DBP in the Advantage system. Thus, the Liaison is a true RIA method. Details on this procedure are available elsewhere.<sup>11</sup> The Liaison 25(OH)D assay is cospecific for 25(OH)D<sub>2</sub> and 25(OH)D<sub>3</sub>, so it reports a "total" 25(OH)D concentration. DiaSorin recently introduced a second-generation Liaison 25(OH)D assay. This new version has increased functional sensitivity and much improved assay precision. The Liaison 25(OH)D assay is the single most widely used 25(OH)D assay in the world for clinical diagnosis.

The most recent addition to the automated 25(OH)D assay platforms is from Roche Diagnostics. Their test is an RIA called vitamin D<sub>3</sub>(25-OH), which can be performed on their Elecsys and Cobas systems. Roche only released this assay in 2007, so very little information on it is available. However, the assay can only detect 25(OH)D<sub>3</sub>, so it will not be a viable product in countries in which vitamin D<sub>2</sub> is used clinically, including the United States.<sup>12</sup>

### **Direct Physical Detection Methods**

Direct detection methodologies for determining circulating 25(OH)D include both HPLC and LC/MS procedures.<sup>13–17</sup> The HPLC methods separate and quantitate circulating 25(OH)D<sub>2</sub> and 25(OH)D<sub>3</sub> individually. HPLC followed by UV detection is highly repeatable and, in general, most people consider it the gold standard method. However, these methods are cumbersome and require a relatively large sample as well as an internal standard. Sample throughput is slow and is not suited to a high-demand clinical laboratory processing up to 10,000 25(OH)D assays per day.

Researchers have recently revitalized LC/MS as a viable method to assess circulating 25(OH)D.<sup>14–17</sup> As with HPLC, LC/MS quantitates 25(OH)D<sub>2</sub> and 25(OH)D<sub>3</sub> separately. When performed properly, LC/MS is a very accurate testing method. However, the equipment is very expensive and its overall sample throughput cannot, when performed properly, match that of the automated instrumentation format. As a methodology, LC/MS can compare favorably with RIA techniques.<sup>15,16</sup> One unique problem with LC/MS is its relative inability to discriminate between 25(OH)D<sub>3</sub> and its inactive isomer 3-epi-25(OH)D<sub>3</sub>. This problem has been especially noticeable in the circulation of newborn infants.<sup>14</sup> Next to the DiaSorin assays, LC/MS is the next most used procedure for the clinical assessment of circulating 25(OH)D.

### **DETERMINING ANALYTICAL RECOVERY OF 25(OH)D<sub>2</sub> AND 25(OH)D<sub>3</sub> IN HUMAN SERUM OR PLASMA**

Questions constantly arise regarding the various 25(OH)D assay procedures' ability to accurately measure total 25(OH)D (25(OH)D<sub>2</sub> + 25(OH)D<sub>3</sub>) levels in human samples.<sup>8</sup> A brief study recently has described the ability of the DiaSorin Liaison Total-D 25(OH)D Assay System to perform this task as compared with the gold standard HPLC/UV quantitation of 25(OH)D<sub>2</sub> and 25(OH)D<sub>3</sub>.<sup>18</sup> Baseline serum samples that contained only 25(OH)D<sub>3</sub> were obtained from 9 volunteers. All subjects then consumed 50,000 IU/d vitamin D<sub>2</sub> for a period of 14 days. Seven days following the final dose serum samples were again obtained. For exogenous in vitro recovery experiments 32 ng/mL of either 25(OH)D<sub>2</sub> or 25(OH)D<sub>3</sub> were added, in a small volume of ethanol, to each baseline serum sample. All samples were then subjected to direct HPLC/UV quantitation to determine individual levels of 25(OH)D<sub>2</sub> and 25(OH)D<sub>3</sub><sup>9</sup> or the DiaSorin Liaison Total-D Assay.

25(OH)D calibrators from The National Institute of Standards and Technology (NIST) were also tested. NIST describes the samples as Level 1, "normal" human serum; Level 2, "normal" human serum diluted 1:1 with horse serum; and Level 3, "normal" human serum "spiked" with 25(OH)D<sub>2</sub> attempting to equal the amount of endogenous 25(OH)D<sub>3</sub> contained in the sample. Horse serum from Sigma Chemical Company was also accessed.

In the group of volunteers the baseline total 25(OH)D was  $48.3 \pm 19.0$  and  $43.7 \pm 16.8$  ng/mL ( $\bar{x} \pm SD$ ) by HPLC-UV and Liaison, respectively. In these baseline samples HPLC-UV analysis demonstrated 99% of the circulating 25(OH)D to be of the D<sub>3</sub> form, and only 2 of 9 subjects had detectable (>1.0 ng/mL) 25(OH)D<sub>2</sub>. Following 14 days of

oral vitamin D<sub>2</sub> supplementation, total 25(OH)D levels were determined to be  $81.1 \pm 21.9$  and  $80.0 \pm 25.5$  ng/mL by HPLC-UV and Liaison, respectively. By HPLC analysis the elevations in 25(OH)D<sub>2</sub> ranged from 25 to 88 ng/mL. In these postsupplementation samples, HPLC-UV analysis also revealed 25(OH)D<sub>3</sub> to be 43.5% of the total while the remaining 56.5% was 25(OH)D<sub>2</sub>. The regression relationship of pre and post samples between HPLC-UV and Liaison was  $\text{Liaison Total-D} = 1.04 (\text{HPLC-UV}) - 5.27$ ,  $r^2 = 0.95$  (Fig. 1). The recovery of exogenously added 25(OH)D<sub>2</sub> or 25(OH)D<sub>3</sub> to baseline samples was  $98.3\% \pm 5.7\%$  and  $99.0\% \pm 6.7\%$ , respectively by HPLC-UV analysis, and  $22.8\% \pm 19.7\%$  and  $62.7\% \pm 24.8\%$ , respectively by Liaison analysis.<sup>18</sup>

NIST Level 1 concentrations measured by the Liaison compared favorably with HPLC results. However, NIST Level 2 was higher (Liaison vs HPLC) and Level 3 was lower (Liaison vs HPLC). The higher concentration in the NIST Level 2 can be attributed to the impact of the horse serum matrix, and lower levels in NIST Level 3 can be attributed to the lack of recovery of exogenous material by the Liaison system.

The data reveal an important artifact that could lead to false conclusions about the ability of direct competitive antibody-based chemiluminescence assays to quantitatively detect 25(OH)D<sub>2</sub> and/or 25(OH)D<sub>3</sub> in patient samples. It has proven difficult to produce an antibody that is cospecific for the detection of 25(OH)D<sub>2</sub> and 25(OH)D<sub>3</sub> in human serum. In fact, only one such antibody has been reported, being the antibody used in the DiaSorin 25(OH)D assays.<sup>9</sup>

In the United States it is imperative that any 25(OH)D assay used for clinical diagnosis has the ability to detect total 25(OH)D, a sum of 25(OH)D<sub>2</sub> and 25(OH)D<sub>3</sub>. With a single exception, all competitive protein-binding assays introduced commercially have discriminated against 25(OH)D<sub>2</sub> including the now defunct Nichols Advantage 25(OH)D assay system. It is also a fact that approximately 99% of the United States population has undetectable 25(OH)D<sub>2</sub> in their circulation, because vitamin D<sub>2</sub> is rarely

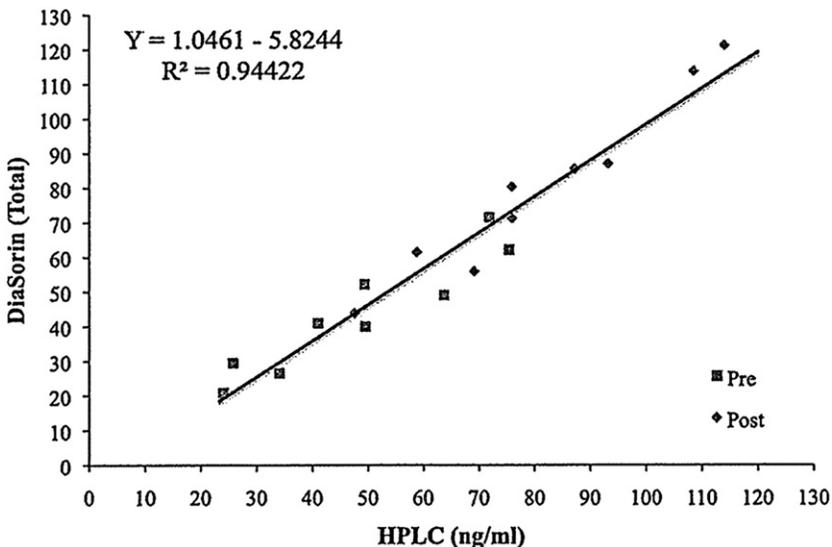


Fig. 1. Elevations in plasma total 25(OH)D in volunteers following supplementation with vitamin D<sub>2</sub> as measure by the DiaSorin Liaison Total method versus HPLC. Volunteers were given vitamin D<sub>2</sub>. Presupplementation concentrations are represented by the closed boxes and postsupplementation by the closed diamonds.

used as a supplement nowadays and patients only receive it when being treated for vitamin D deficiency by a physician. Because blood samples in the general population rarely contain significant amounts of 25(OH)D<sub>2</sub>, and because the compound is usually discriminated against by most antibody-based assays, it is the compound most often added exogenously to human serum to assess cross-reactivity and determine analytical recovery.

We have assumed since the early 1970s that when one adds exogenous 25(OH)D to a blood sample it rapidly binds to its carrier protein, the DBP, with little interaction to other blood components.<sup>19</sup> Up to this point in vitamin D assay technology, exogenous addition of 25(OH)D<sub>2</sub> or 25(OH)D<sub>3</sub> has served clinicians well in the testing of quantitative analytical recoveries of these compounds.<sup>9</sup> Problems were never encountered because extraction procedures were based on organic solvents of one kind or another, and they all destroyed the DBP and liberated the 25(OH)D into solution. The direct serum or plasma assays emerging today do not destroy the carrier proteins. Instead they rely on pH changes and/or blocking agents that liberate the 25(OH)D from its carrier protein but do not affect the ability of the steroid to bind to a specific antibody. This later disruption method is the one employed in the Liaison assay.<sup>11</sup>

The results clearly demonstrate that exogenously added 25(OH)D<sub>2</sub> or 25(OH)D<sub>3</sub> do not distribute themselves on the DBP as occurs when assembled in vivo. The other possibility is that exogenously added 25(OH)D distribute to moieties other than the DBP, suggested by the clear linear relationship observed from in vivo human samples containing elevated amounts of 25(OH)D<sub>2</sub> when assayed by the Liaison method versus HPLC-UV. On the other hand, the failure of quantitative recovery is apparent from exogenously added 25(OH)D<sub>2</sub> or 25(OH)D<sub>3</sub> to the same samples when the assay methods are compared (**Table 1**). This study describes an in vitro anomaly that really has no physiologic relevance, but could result in erroneous conclusions about 25(OH)D assay performance when comparing sample destruction methods such as HPLC-UV versus the newer sample disruption method such as the Liaison assay.<sup>18</sup> Extreme caution is warranted when preparing samples for such comparisons, as is being done by the Vitamin D External Quality Assessment Scheme (DEQAS) and NIST.

<b>Table 1</b>		
<b>Comparison of 25(OH)D concentrations measured by the DiaSorin Liaison and HPLC as a result of various exogenous and endogenous treatments.</b>		
<b>Sample ID</b>	<b>DiaSorin Liaison</b>	<b>HPLC</b>
	<b>Total 25(OH)D (ng/mL)</b>	
Baseline	43.7 ± 16.8	48.3 ± 19.0
Vitamin D <sub>2</sub>	81.1 ± 21.9	80.0 ± 23.5
Baseline+25(OH)D <sub>2</sub> <sup>a</sup>	51.0 ± 16.8 (22.8%)	79.7 ± 19.0 (98.3%)
Baseline+25(OH)D <sub>3</sub> <sup>a</sup>	63.7 ± 20.4 (62.7%)	80.0 ± 18.5 (99.0%)
Horse serum	12.7 ± 1.0	4.7 ± 0.2
NIST Level 1 [22–24] <sup>b</sup>	24.4 ± 0.8 (106%)	26.0 ± 1.1 (113%)
NIST Level 2 [12–14] <sup>b</sup>	19.8 ± 0.5 (152%)	15.9 ± 0.7 (122%)
NIST Level 3 [42–46] <sup>b</sup>	27.2 ± 1.0 (61.8%)	48.1 ± 3.0 (109%)

<sup>a</sup> 32 ng/mL was added to each of 9 samples. Values in parentheses represent amount of 25(OH)D recovered as a percentage of mean values.

<sup>b</sup> Values in brackets are expected values provided by NIST. Values in parentheses represent amount of 25(OH)D recovered as a percentage of mean values.

## DETERMINING AND DEFINING A "NORMAL" CIRCULATING 25(OH)D LEVEL

To define a "normal" circulating level of a given substance or nutrient, one usually obtains blood samples from a diverse population, measures the substance in question, plots the data by Gaussian distribution, and determines normality. This method works well for nutrients such as folate or vitamin E, and was precisely how normative circulating levels of 25(OH)D were defined in humans beginning about 40 years ago by Haddad and Chyu,<sup>3</sup> who sampled a population of "normal" individuals whom were asymptomatic for disease, assessed circulating 25(OH)D, and determined a mean value. In their study Haddad and Chyu also assessed 25(OH)D in a group of lifeguards and demonstrated their levels to be 2.5 times those of the "normals". Countless similar studies performed over the ensuing decades reiterated the same conclusion. The author, however, interpreted the original Haddad data differently, suggesting that the 25(OH)D levels in the lifeguards were normal and the "normals" were actually vitamin D deficient.<sup>20</sup> This interpretation has largely been validated by the current research.

For all practical purposes, vitamin D does not naturally occur in foodstuffs that humans eat. There are exceptions such as oily fish and fish liver oil. In fact, from an evolutionary standpoint, humans did not require vitamin D in their food supply because over millions of years humans evolved a photosynthetic mechanism in their skin to produce large amounts of vitamin D<sub>3</sub>. Thus, our skin is part of the vitamin D endocrine system, and vitamin D<sub>3</sub> is really a prohormone. The problem now is that humans avoid the sun, wear sunscreen, and reside in latitudes for which they are not programmed to live. To make matters worse, the dietary requirement for vitamin D in adults is 200 IU/d, as defined by the Adequate Intake (AI) by the Food and Nutrition Board, and is essentially meaningless.<sup>21</sup> As a result of these factors, a "normal" circulating 25(OH)D range is now defined using various biomarkers of physiology or disease as opposed to a random population Gaussian distribution.

The first use of biomarkers to define "normal" 25(OH)D levels, of course, started with parameters that affected skeletal integrity such as parathyroid hormone, bone mineral density, and intestinal calcium absorption.<sup>20</sup> These parameters demonstrated that a minimum circulating level of 25(OH)D should be at least 32 ng/mL (80 nmol).<sup>20,22</sup> At present, the "normal" circulating 25(OH)D level also relies on data based on the other diverse physiologic functions of 25(OH)D including cancer prevention,<sup>23-31</sup> infectious disease,<sup>32-37</sup> cardiovascular health,<sup>38-45</sup> diabetes,<sup>45-48</sup> and autoimmune control.<sup>49-51</sup> Because of the diverse interaction of vitamin D with our genome, this list is certain to grow.<sup>52</sup> For the present it is generally agreed that a normal level of circulating 25(OH)D is 32 to 100 ng/mL (80-250 nmol). It must be noted that 32 ng/mL is not an "optimum" level but a minimum "normal" level. What constitutes an "optimum" level remains to be determined, and may well be different for varied physiologic processes.

## CLINICAL REPORTING OF CIRCULATING 25(OH)D CONCENTRATIONS

As highlighted earlier, all DiaSorin 25(OH)D assays are approved by the FDA for clinical utility. Thus, the diagnostic 25(OH)D tests sold by DiaSorin and IDS Diagnostics (Fountain Hills, AZ, USA) are under strict FDA control and monitoring for assay performance and reliability. In what is considered a distributing trend, many clinical reference laboratories are replacing these FDA-approved tests with "home-brew" LC/MS methods that are diverse and not under FDA scrutiny. The reasons for this switch in use are the "perceived" advantages of LC/MS technology being more accurate, precise, specific, cost effective, and providing the separate determination of 25(OH)D<sub>2</sub> and 25(OH)D<sub>3</sub>. First, with respect to accuracy and precision, the DiaSorin and IDS RIA methods

perform at least as well as LC/MS methods according to the DEQAS operated out of London, United Kingdom. As far as specificity goes, the DiaSorin tests appear more specific than LC/MS methodology in that the DiaSorin assays do not detect the inactive 3-epimer of 25(OH)D<sub>3</sub>.<sup>14</sup> Finally, LC/MS assays are marketed on their ability to separately measure 25(OH)D<sub>2</sub> and 25(OH)D<sub>3</sub> in a blood sample. However, clinically there is no advantage to this separate measurement claim. Not a single scientific publication exists that demonstrates separate 25(OH)D<sub>2</sub> and 25(OH)D<sub>3</sub> whose measurements are superior to a “total” 25(OH)D value as supplied by the DiaSorin tests. In fact, this separate reporting has been shown to confuse clinicians.<sup>53</sup> The truth is that LC/MS laboratories report separate values because that is how LC/MS technology has to report the data,<sup>14–17</sup> and is not a reason to “spin” it to a clinical advantage. Also, the FDA has made its opinion on the separate reporting of 25(OH)D<sub>2</sub> and 25(OH)D<sub>3</sub> clear. In 2007, ESA Biosciences Inc (Chelmsford, MA, USA) submitted a 510K application to the FDA seeking approval of an HPLC procedure to determine 25(OH)D<sub>2</sub> and 25(OH)D<sub>3</sub> separately in blood. The FDA responded “Assays intended for clinical use must have a clinical indication as well as an analytical claim. Please provide additional information to establish the clinical validity of separate 25(OH)D<sub>2</sub> and 25(OH)D<sub>3</sub> in diagnosis.” Of course, no such data exist so ESA’s test was only approved after they agreed to report only “total” 25(OH)D. Thus, separate reporting of 25(OH)D<sub>2</sub> and 25(OH)D<sub>3</sub> in the eyes of the FDA is of no medical advantage or use. Some LC/MS laboratories have actually billed inappropriate Current Procedural Terminology codes to enhance return for these separate reported values. The author considers this practice to be abusive and fraudulent, and believe it must end. Further, 99% of all patient samples assayed will not contain any 25(OH)D<sub>2</sub>.

Replacement of FDA-controlled devices such as the DiaSorin and IDS assays with “home-brew” LC/MS assays from a clinical diagnostic standpoint is, again, disturbing, because the DiaSorin assays have and continue to be the standard of clinical 25(OH)D assessment. One can say this because the “normal” range of circulating 25(OH)D is almost entirely based on clinical studies using the DiaSorin tests. In fact, Labcorp (Burlington, NC, USA) uses a publication by Hollis<sup>20</sup> on which to base its clinical range of 25(OH)D levels. In turn, this publication is based on DiaSorin assay-based clinical studies, so unless a given LC/MS method is calibrated against the DiaSorin methods, this reference range should not be reported against.

Many years and clinical studies have gone into establishing the DiaSorin reference range and, as stated earlier, this consists of thousands of scientific publications. To prove the point the author has selected some large significant clinical studies on which the “normal” circulating level of 25(OH)D is based, most of which used DiaSorin and some IDS assays as their method of analysis. No LC/MS clinical studies are included because basically none exist, which is the author’s point exactly.

The DiaSorin RIA has been used to generate all of the 25(OH)D data from the third National Health and Nutrition Examination Survey (NHANES III). Selected references on this topic are included to validate this claim.<sup>38,48,53–56</sup> Many more studies from NHANES exist with respect to vitamin D and all use the DiaSorin RIA. Studies from the huge National Institutes of Health (NIH)-sponsored Women’s Health Initiative (WHI) used the DiaSorin LIAISON assay for the first 2 major publications,<sup>31,57</sup> with others to follow.

The Harvard-based studies, the Health Professionals’ Follow-up Study (HPFAS), and the Nurses’ Health Study (NHS) have been used to establish much of the information in the last decade regarding the relationship of circulating 25(OH)D levels and various disease states such as cancer, autoimmune, cardiovascular, and renal. All of these studies again used DiaSorin-based assays.<sup>24–30,34,35,45–49</sup> Of course, one cannot forget the relationship of vitamin D status, parathyroid hormone, and skeletal

integrity. Hundreds of articles have been published on this topic; most using DiaSorin assays and none using LC/MS testing.

What then should LC/MS laboratories do? If they are going to use the current DiaSorin-based reference range<sup>20</sup> they had better target their values to that of the DiaSorin test. In fact, this is basically how the FDA has been approving new devices for 25(OH)D assessment through the 510K process since the DiaSorin RIA was the first device approved in 1993. The alternative is that each LC/MS site establish their own reference range, which will take years of clinical study because a normal Gaussian distribution is useless in establishing a normative 25(OH)D range. In fact, this “normalization” of values is common between other 25(OH)D assays and DiaSorin testing, as recent articles demonstrate.<sup>58</sup>

Finally, clinical reference laboratories should simply use a single reference range to report circulating 25(OH)D levels, as does Labcorp, 32 to 100 ng/mL. Compare this to the Mayo Clinic, which reports 4 different “classes” of 25(OH)D status. This type of reporting is confusing and should be discontinued.

## METHODS OF 1,25(OH)<sub>2</sub>D QUANTITATION

Of all the steroid hormones, 1,25(OH)<sub>2</sub>D represented the most difficult challenge to the analytical biochemist with respect to quantitation. 1,25(OH)<sub>2</sub>D circulates at picomolar concentrations (too low for direct UV or MS quantitation), is highly lipophilic, and its precursor, 25(OH)D, circulates at nanomolar levels. The development of simple, rapid assay for this compound has proven to be a daunting task.

### *Radioreceptor Assays*

The first radioreceptor assay (RRA) for 1,25(OH)<sub>2</sub>D was introduced in 1974.<sup>59</sup> Although this initial assay was extremely cumbersome, it did provide invaluable information with respect to vitamin D homeostasis. This initial RRA required a 20-mL serum sample, which was extracted using Bligh-Dyer organics. The extract had to be purified by 3 successive chromatographic systems, and chickens had to be euthanized and the vitamin D receptor (VDR) harvested from their intestines. By 1976, the volume requirement for this RRA had been reduced to a 5-mL sample, and sample prepurification had been modified to include HPLC.<sup>60</sup> However, the sample still had to be extracted using a modified Bligh-Dyer procedure and then prepurified on Sephadex LH-20. Chicken intestinal VDR was still used as a binding agent.

A major advancement occurred in 1984 with the introduction of a radically new concept for the RRA determination of circulating 1,25(OH)<sub>2</sub>D.<sup>61</sup> This new RRA used solid-phase extraction of 1,25(OH)<sub>2</sub>D from serum along with silica cartridge purification of 1,25(OH)<sub>2</sub>D. As a result, the need for HPLC sample prepurification was eliminated. Also, this assay used VDR isolated from calf thymus, which proved to be quite stable and thus had to be prepared only periodically. Further, the volume requirement was reduced to 1 mL of serum or plasma. This assay opened the way for any laboratory to measure circulating 1,25(OH)<sub>2</sub>D. This procedure also resulted in the production of the first commercial kit for 1,25(OH)<sub>2</sub>D measurement. This RRA was further simplified in 1986 by decreasing the required chromatographic purification steps.<sup>62</sup> This method has become a citation classic.<sup>63</sup>

As good as the calf thymus RRA for 1,25(OH)<sub>2</sub>D was, it still possessed 2 serious shortcomings. First, VDR had to be isolated from thymus glands. Second, because the VDR is so specific for its ligand, only <sup>3</sup>H-1,25(OH)<sub>2</sub>D<sub>3</sub> could be used as a reporter, eliminating the use of <sup>125</sup>I or chemiluminescent reporter; this was a major handicap, especially for the commercial laboratory.

### **Radioimmunoassay**

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In 1978, the first RIA for 1,25(OH)<sub>2</sub>D was introduced.<sup>64</sup> Although it was an advantage not to have to isolate the VDR as a binding agent, this RIA was relatively nonspecific, so the cumbersome sample preparative steps were still required. Over the next 18 years all RIAs developed for 1,25(OH)<sub>2</sub>D suffered from the same shortcomings. In 1996, the author's group developed the first significant advance in 1,25(OH)<sub>2</sub>D quantification in a decade.<sup>65</sup> This RIA incorporated a <sup>125</sup>I reporter, as well as standards in an equivalent serum matrix, so individual sample recoveries were no longer required. The sample purification procedure is the same one previously used for the rapid RRA procedure.<sup>62</sup> This assay has 100% cross-reactivity between 1,25(OH)<sub>2</sub>D<sub>2</sub> and 1,25(OH)<sub>2</sub>D<sub>3</sub> and is FDA-approved for clinical diagnosis in humans.

Another <sup>125</sup>I-based RIA for 1,25(OH)<sub>2</sub>D is also commercially available from IDS Ltd. The basis of this kit is a selective immunoextraction of 1,25(OH)<sub>2</sub>D from serum or plasma with a specific monoclonal antibody bound to a solid support. This antibody is directed toward the H-hydroxylated A ring of 1,25(OH)<sub>2</sub>D.<sup>66</sup> This assay procedure has never been published in detail, so critical evaluation is difficult. The author concluded that this immunoextraction procedure was highly specific for the 1-hydroxylated forms of vitamin D. However, he also believes that this procedure overestimated circulating 1,25(OH)<sub>2</sub>D levels. Evidence of this overestimation is evident in a recent publication that shows a correlation of circulating 25(OH)D and 1,25(OH)<sub>2</sub>D at physiologic levels,<sup>67</sup> indicating that 25(OH)D may be interfering with the assay.

Enzyme-linked immunosorbent assays for circulating 1,25(OH)<sub>2</sub>D determinations do exist commercially from Immunodiagnostik and IDS. However, their performance has never been published in detail.

### **Direct Physical Detection Methods**

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Direct detection methodology for determining circulating 1,25(OH)<sub>2</sub>D is very problematic because of the low concentration (pmol) in the blood. Because of this fact, direct UV detection is not possible. Recently 2 commercial laboratories, Mayo Clinic and Quest Diagnostics, have begun to offer LC/MS detection of circulating 1,25(OH)<sub>2</sub>D. However, details of these analyses are not in the public domain.

### **DETERMINING AND DEFINING A "NORMAL" CIRCULATING 1,25(OH)<sub>2</sub>D LEVEL**

Unlike 25(OH)D, a normal circulating level of 1,25(OH)<sub>2</sub>D can be determined from a Gaussian distribution of subjects. Over the last 3 decades this has been accomplished, and a normal adult level has been defined as 16 to 56 pg/mL with a mean of 37.6 pg/mL.<sup>68</sup> Circulating 1,25(OH)<sub>2</sub>D is diagnostic for several clinical conditions, including vitamin D-dependent rickets types I and II, hypercalcemia associated with sarcoidosis, and other hypercalcemic disorders causing increased 1,25(OH)<sub>2</sub>D levels. These other disorders include tuberculosis, fungal infections, Hodgkin disease, lymphoma, and Wegener granulomatosis. In all other clinical conditions involving the vitamin D endocrine system, including hypoparathyroidism, hyperparathyroidism, and chronic renal failure, the assay of 1,25(OH)<sub>2</sub>D is a confirmatory test. It is also important to remember that circulating 1,25(OH)<sub>2</sub>D provides essentially no information with respect to the patient's nutritional vitamin D status. Thus, circulating 1,25(OH)<sub>2</sub>D should not be used as an indicator for hypo- or hypervitaminosis D when nutritional factors are suspected.

A very interesting condition that has a profound effect on circulating 1,25(OH)<sub>2</sub>D levels is pregnancy.<sup>69</sup> During pregnancy, circulating 1,25(OH)<sub>2</sub>D increases dramatically. In the author's recently completed NIH-funded pregnancy and vitamin D trial,

circulating 1,25(OH)<sub>2</sub>D levels averaged 130 pg/mL with levels of 300 to 400 pg/mL observed in some patients. These levels are so dramatic they could almost be used as a pregnancy test. The physiologic purpose of this dramatic elevation remains to be determined.

### STABILITY OF 25(OH)D AND 1,25(OH)<sub>2</sub>D IN SERUM OR PLASMA

Researchers have known for nearly 30 years that endogenous 25(OH)D and 1,25(OH)<sub>2</sub>D are extremely stable in serum or plasma.<sup>70</sup> Lissner and colleagues<sup>70</sup> showed that vitamin D metabolites in blood stored at 24°C for up to 72 hours 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.<sup>71</sup> The author has used the same pooled human 25(OH)D and 1,25(OH)<sub>2</sub>D internal controls stored at -20°C for more than 10 years with no detectable degradation of either compound. The 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)<sub>2</sub>D values.

The author has performed experiments with the aim of destroying endogenous 25(OH)D and 1,25(OH)<sub>2</sub>D in plasma to obtain a vitamin D-free human plasma to prepare various immunoassay procedure calibrators. When crystalline 25(OH)D<sub>3</sub> or 1,25(OH)<sub>2</sub>D<sub>3</sub> was placed in ethanol in an open glass Petri dish and the dish exposed to intense UV light, the UV light destroyed the compounds within a few minutes. When the same experiment was conducted using serum or plasma, however, the 25(OH)D<sub>3</sub> and 1,25(OH)<sub>2</sub>D<sub>3</sub> levels did not change after 2 days of UV light exposure. The author 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)<sub>2</sub>D are very stable in serum or plasma, so they require only minimal attention to storage conditions.

### STANDARDIZATION OF 25(OH)D AND 1,25(OH)<sub>2</sub>D ANALYSIS

DEQAS (Internet: [www.deqas.org](http://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 approximately 600 participating laboratories worldwide. The organization's major aim today is to assess the analytical reliability of 25(OH)D and 1,25(OH)<sub>2</sub>D 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 gas chromatography-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
- Providing a forum for exchanging information on all aspects of vitamin D assay methodology.

The author's laboratory has participated in both the DEQAS 25(OH)D and DEQAS 1,25(OH)<sub>2</sub>D survey since 1997, and the survey has been invaluable in maintaining the integrity of his assay procedure. 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)D<sub>2</sub> concentrations.<sup>8</sup> 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, it is hoped that DEQAS can incorporate the new NIST 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.

## SUMMARY

The assessment of circulating 25(OH)D and, to a lesser degree, 1,25(OH)<sub>2</sub>D is rapidly becoming an important clinical tool in the diagnosis and management of many diverse pathologies. At present, the reference ranges for circulating 25(OH)D and 1,25(OH)<sub>2</sub>D are 32 to 100 ng/mL and 16 to 56 ng/mL, respectively, and are largely based on clinical data derived from the FDA-cleared DiaSorin assay procedures.

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