

Assessment of vitamin D status and definition of a normal circulating range of 25-hydroxyvitamin D

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Purpose of review

The assessment of circulating 25-hydroxyvitamin D [25(OH)D] for clinical diagnosis has increased in an exponential fashion during the past 5 years. It is thus timely to review the reasons for this increase as well as the diverse analytical methods used to meet this need.

Recent findings

Nutritional vitamin D status, as defined by circulating levels of 25(OH)D, has long been implicated in skeletal health. However, in the past decade circulating 25(OH)D has been strongly linked in humans to cancer rates, autoimmune disease, cardiovascular health and infectious disease. As a result, availability and rapid analytical turnaround of 25(OH)D assays have had to improve. Today these demands are largely met in the clinical laboratory by direct automated chemiluminescent platform analysis or high-throughput LC/MS procedures. These methods are diverse and often do not agree with respect to designated reference ranges.

Summary

The assessment of circulating 25(OH)D levels has become an important clinical tool in the management and prevention of diverse disease states. For this reason, assay standardization as well as a uniform reference range for circulating 25(OH)D levels must be achieved.

Keywords

25-hydroxyvitamin D, cancer, cardiovascular disease, infectious disease, reference range, skeletal integrity, vitamin D

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Introduction

What is a normal circulating level of 25(OH)D indicative of sufficient levels of 25(OH)D to meet all physiological needs, not simply skeletal in humans? Until very recently, this was addressed by simply gathering a diverse population of individuals who were asymptomatic for disease, measuring circulating 25(OH)D, and plotting the data using a Gaussian distribution. This is how Haddad and Chyu [1] performed their assessment of 25(OH)D status nearly four decades ago. Surprisingly, this is exactly how 'normative' 25(OH)D levels were reported to clinicians up until about three years ago. During this period the 'normal' 25(OH)D range was 10–80 ng/ml. So basically, if one had a heartbeat, one had a 'normal' circulating 25(OH)D level. Fortunately, for the health of us all, a 'normal' circulating 25(OH)D is now defined as 32–100 ng/ml. How and why this dramatic change occurred will be discussed in this text.

Vitamin D structure and chemistry

Vitamin D is a 9,10-seco steroid and exists in two distinct forms: vitamin D₂ and vitamin D₃. Vitamin D₂ is a 28-carbon molecule derived from the plant sterol ergosterol, whereas vitamin D₃ is a 27-carbon derivative of cholesterol. Vitamin D₂ differs from vitamin D₃ in that it contains an extra methyl group and a double bond between carbons 22 and 23.

The most important aspect of vitamin D chemistry centers 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 vitamin D. Research has now demonstrated that vitamin D₂ is much less bioactive than vitamin D₃ in humans [2,3] although a recent study disputes this finding [4]. The parent

compounds, vitamins D₂ and D₃ 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 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. This metabolic activation and inactivation are well characterized and result in a plethora of vitamin D metabolites [5]. Of these metabolites, only 25(OH)D and 1,25-dihydroxyvitamin D provide any clinically relevant information. 25(OH)D₂ and 25(OH)D₃ are commonly known as calcifediol and the 1,25(OH)₂D metabolites as calcitriol. In this review I will focus entirely on 25(OH)D analysis since it is the metabolite that supplies, by far, the most clinically relevant information.

Methods of 25(OH)D quantitation

The assessment of circulating 25(OH)D started its journey approximately four decades ago with the advent of the competitive protein-binding assay (CPBA) [1]. 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). I will provide a brief description of each technique.

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 CPBA for 25(OH)D. Haddad and Chyu [1], introduced this CPBA almost four decades ago. The assay assessed circulating 25(OH)D concentrations using the vitamin D-binding protein (DBP) as a primary binding agent and ³H-25(OH)D₃ 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 two forms, 25(OH)D₂ and 25(OH)D₃, 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 [6]. The original Haddad procedure overcame the matrix problem by using chromatographic sample purification before CPBA [1].

Researchers had a strong desire to simplify this cumbersome CPBA for 25(OH)D, so Belsey *et al.* [7] 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 ³H-25(OH)D₃. However, after several years of trying, researchers were unable to validate the Belsey assay due to matrix problems originating from ethanolic sample extraction [8].

The 25(OH)D CPBA's did have the advantage of being cospecific for 25(OH)D₂ and 25(OH)D₃ and thus provided a 'total' 25(OH)D value if the assay was valid. The DBP's binding cospecificity 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, 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)D₂ [9,10]. Although never described, these problems were probably linked to the DBP's inability to resolve the matrix problems associated with direct sample assay. Currently, 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 CPBA's.

Radioimmunoassay

In the early 1980's, my group decided that a nonchromatographic RIA for circulating 25(OH)D would be the best approach to measuring the substance. We therefore designed an antigen that would generate an antibody that was cospecific for 25(OH)D₂ and 25(OH)D₃ [11]. In addition, we designed a simple extraction method that allowed simple nonchromatographic quantification of circulating 25(OH)D [11]. In 1985 Immunonuclear Corp., now known as DiaSorin, introduced this ³H-based RIA as

a kit on a commercial basis. This RIA was further modified in 1993 to incorporate a ^{125}I -labeled reporter and calibrators (standards) in a serum matrix [12]. 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 these past 23 years, these DiaSorin tests have been utilized 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

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 [13]. 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)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.* [7]. 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 [13]. 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 [13]. The Liaison 25(OH)D assay is cospecific for 25(OH)D₂ and 25(OH)D₃, 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 D₃(25-OH) and it 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)D₃, so it will not be a viable product in countries in which vitamin D₂ is used clinically, including the USA.

Direct physical detection methods

Direct detection methodologies for determining circulating 25(OH)D include both HPLC and LC/MS procedures [14–18]. 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 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 [15–18]. As with HPLC, LC/MS quantitates 25(OH)D₂ and 25(OH)D₃ 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 [16–18]. One unique problem with LC/MS is its relative inability to discriminate between 25(OH)D₃ and its inactive isomer 3-epi-25(OH)D₃. This problem has been especially noticeable in the circulation of newborn infants [15]. Next to the DiaSorin assays, LC/MS is the next most utilized procedure for the clinical assessment of circulating 25(OH)D.

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 [1]. They sampled a population of 'normal' individuals whom were asymptomatic for disease, assessed circulating 25(OH)D and determined a mean value. In their study they also assessed 25(OH)D in a group of lifeguards and demonstrated their levels to be 2.5 times that of the 'normals'. Countless similar studies performed over the ensuing decades reiterated the same conclusion. I,

however, interpreted the original Haddad data differently; I suggested that the 25(OH)D levels in the lifeguards are normal and the 'normals' were actually vitamin D deficient [19]. 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. The fact is, 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 D₃. Thus, our skin is part of the vitamin D endocrine system, and vitamin D₃ is really a preprohormone. The problem now is that humans avoid the sun, wear sunscreen and reside in latitudes that we 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 [20]. As a result of these factors, we now define a 'normal' circulating 25(OH)D range 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 [21–25]. These parameters demonstrated that a minimum circulating level of 25(OH)D should be at least 32 ng/ml (80 nmol) [19,26]. Presently, the 'normal' circulating 25(OH)D level also relies on data based on the other diverse physiological function of 25(OH)D including cancer prevention [27*,28–35], infectious disease [36–39], cardiovascular health [40,41*–43*], diabetes [44–46] and autoimmune control [47]. Because of the diverse interaction of vitamin D with our genome this list is certain to grow [48]. For the present it is generally agreed that a normal level of circulating 25(OH)D is 32–100 ng/ml (80–250 nmol). Please take note 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 different physiological 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, Arizona, USA) are under strict FDA control and monitoring for assay performance and reliability. In what we consider 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 utilization are the 'perceived' advan-

tages of LC/MS technology being more accurate, precise, specific, cost effective and providing the separate determination of 25(OH)D₂ and 25(OH)D₃. 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 Vitamin D External Quality Assessment Scheme (DEQAS) operated out of London, UK. 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)D₃ [15]. Finally, LC/MS assays are marketed on their ability to separately measure 25(OH)D₂ and 25(OH)D₃ in a blood sample. Clinically, however, there is no advantage to this separate measurement claim. Not a single scientific publication exists that demonstrates separate 25(OH)D₂ and 25(OH)D₃ 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 the clinician [49]. The truth is, LC/MS laboratories report separate values because that is how LC/MS technology has to report the data [15–18] and is not a reason to 'spin' it into a clinical advantage. Some LC/MS laboratories have actually billed inappropriate CPT codes to enhance return for these separate reported values. We consider this practice to be abusive and fraudulent and feel it must end. Further, 99% of all patient samples assayed will not contain any 25(OH)D₂.

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. It is disturbing because the DiaSorin assays have and continue to be the standard of clinical 25(OH)D assessment. We 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, North Carolina, USA) uses a publication by Hollis [19] on which to base their 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 we stated earlier, this consists of thousands of scientific publications. To prove our point we have selected some large significant clinical studies on which the 'normal' circulating level of 25(OH)D is based, most of which utilized DiaSorin and some IDS assays as their method of analysis. I have not included any LC/MS clinical studies because basically none exist, which is my 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). We have included selected references on this topic to validate our claim [28–40,41*–43*,44–46,47,50]. Many more studies from NHANES exist with respect to vitamin D and all use the DiaSorin RIA. Studies from the huge NIH sponsored Women's Health Initiative (WHI) used the DiaSorin LIAISON assay for the first two major publications [35,51] with others to follow.

The Harvard-based studies, the Health Professionals' Follow-up Study (HPFS) and the Nurses' Health Study (NHS) have been used to establish much of the information in the last decade with regard to the relationship of circulating 25(OH)D levels and various disease states such as cancer, autoimmune, cardiovascular and renal. All of these studies again utilized DiaSorin-based assays [28–40,41*–43*,44–46,47]. Of course, we cannot forget the relationship of vitamin D status, PTH and skeletal integrity. Hundreds of papers have been published on this topic; most using DiaSorin assays none using LC/MS testing.

What then should LC/MS laboratories do? If they are going to use the current DiaSorin-based reference range [19] they had better target their values to that of the DiaSorin test. In fact, this is basically how the FDA approves new devices for 25(OH)D assessment through the 510 K 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 since a normal Gaussian distribution is useless in establishing a normative 25(OH)D range. In fact, this 'normalization' of values is quite common between other 25(OH)D assays and DiaSorin testing as recent articles demonstrate [17,52]. For instance, if a recently published LC/MS article was used for diagnosis, the levels reported would have to be increased by 13% if the DiaSorin reference range is to be used for clinical diagnosis [17].

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

Conclusion

The assessment of circulating 25(OH)D is rapidly becoming an important clinical tool in the diagnosis and management of many diverse pathologies. At present, the reference range for circulating 25(OH)D is 32–100 ng/ml (80–250 nmol) and is largely based on clinical data derived from the DiaSorin assay procedures.

References and recommended reading

Papers of particular interest, published within the annual period of review, have been highlighted as:

- of special interest
- of outstanding interest

Additional references related to this topic can also be found in the Current World Literature section in this issue (p. 549).

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