

Confusions in Vitamin D Estimation and Interpretation

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Abstract: Role of vitamin D in calcium and phosphorous regulation and in bone metabolism is a well known fact and so, was the need for diagnosing vitamin D deficiency in osteoporosis, osteomalacia and fractures. Discovery of the presence of vitamin D receptor in several tissues has lead to several research works to know the effect of vitamin D on these tissues. This has thrown light on previously blind areas. Vitamin D deficiency is known to be present in cancers, infectious diseases, autoimmune disorders, cardiovascular disease type 2 diabetes mellitus. These findings have resulted in increased laboratory requests for vitamin D estimation. This article aims at providing insight 1. into vitamin D and its metabolic effects, 2. different forms of vitamin D 3. existing methods to estimate vitamin D 4. Selection of the method for estimation, and reference methods for vitamin D estimation, 5. Factors causing variations in vitamin D results, 6. internal and external quality control issues, 7. Interpretation of results.

Keywords: 25-hydroxy D₃, metabolism, 1 α , 25-hydroxy D₃, Method of estimation, standards.

I. Introduction

Vitamin D is a fat soluble vitamin. The status of vitamin D in humans depends on a) dietary intake of vitamin D₃ and b) exposure to UV radiation. A diet high in oily fish is a rich source of vitamin D. The skin produces vitamin D₃, photochemically from the previtamin D, 7-dehydrocholesterol on exposure to sunlight (or) of artificial UV light[1]. The conjugated double bond system in ring B allows the absorption of light at UV range (290-315 nm) resulting in the endogenous production of vitamin D₃.

Metabolism:

Term vitamin D represents D₂ and D₃. D₂ is produced by ergosterol and D₃ by 7-dehydrocholesterol. Vitamin D₂ and D₃ from dietary sources are carried by chylomicrons through lymphatic system into the venous circulation. Vitamin D in the circulation is bound to the vitamin D binding protein. The vitamin D is transported to liver, where by the action of 25-hydroxylase enzyme gets metabolized to 25-OH D₃ vitamin. 25-hydroxyl D₃ vitamin is biologically inactive, and it must be converted to 1 α ,25-(OH)₂ D₃ in the kidneys for its biological activity. In the kidney 25-OH D₃ gets metabolized to its active form by the enzyme 25-OH D 1 α -hydroxylase [2-5]. Another metabolite of 25-OH D is also formed in the kidney by 24-hydroxylation i.e. 24, 25-(OH)₂ D₃. These active metabolites are transported to the distal target organs. There they bind to nuclear receptors, vitamin D binding receptor (VDR) and elicit their appropriate biological responses. Over the past several decades the influence of vitamin D₃ on various tissues is defined by the tissue distribution of VDR. Research HAS SHOWN THAT 1 α ,25-(OH)₂ D₃ initiates the physiologic responses of ≥ 36 cell types that possess the VDR. Apart from endocrine production by the kidney, i.e. circulating, 1 α ,25-(OH)₂ D₃, researchers have found the paracrine production of this steroid hormone in ≥ 10 extrarenal organs[1].

The structure of vitamin D contains steroid nucleus, cycloperhydrophenanthrene ring. As the mechanism of vitamin D action is similar to the action of steroid hormone, it is called as a hormone apart from vitamin. The hormone similar to steroid hormones generates biological responses both by regulating gene transcription, and by rapidly activating a variety of signal transduction pathways at or near the plasma membrane [6]. 1 α ,25-(OH)₂ D₃ can interact with the vitamin D receptor (VDR) localized in the cell nucleus to generate genomic responses and in caveolae with the plasma membrane VDR to generate rapid responses. Binding of 1 α ,25-(OH)₂ D₃ to the membrane VDR may result in the activation of one or more second messenger systems, including phospholipase C, protein kinase C, G-protein coupled receptor or phosphatidylinositol-3-kinase. Some of the second messengers such as RAF/MAP kinase, PI-3,4,5-triphosphate can engage in crosstalk with the nucleus to modulate gene expression. eg. Are pancreatic β cell, adipocytes, vascular smooth muscle, intestine, monocytes and osteoblasts.

Regulation

The renal production of 1 α , 25-(OH)₂ D₃ is tightly regulated by plasma parathyroid hormone levels, serum calcium and phosphorous levels [2-5]. Fibroblast growth factor-23, secreted from the bone and small intestine, also play a role in the regulation [7]. Serum calcium, phosphorous, fibroblast growth factor-23, can

either decrease or increase the renal production of 1, 25- dihydroxyvitamin D₃. This hormone is also regulated by negative feed back. It decreases the synthesis and secretion of parathyroid hormone. 1, 25- dihydroxyvitamin D increases the expression of 25-hydroxy D-24-hydroxylase to catabolise 1,25- dihydroxyvitamin D₃ to the water soluble, biologically inactive calcitric acid which is excreted in bile. 1,25-dihydroxyvitamin D increases intestinal calcium absorption in the small intestine by interacting with the vitamin D receptor-retinoic acid x-receptor complex to enhance the expression of the epithelial calcium channel and calbindin-a calcium binding protein. 1,25- dihydroxy vitamin D is recognized by its receptor in osteoblasts, which increases the expression of the receptor activator of nuclear factor-κB ligand on proosteoclasts, which induces proosteoclasts to become mature osteoclasts. Mature osteoclasts remove calcium and phosphorous from the bone, maintaining calcium and phosphorous levels in the blood. In the presence adequate calcium and phosphorous levels, promote the mineralization of the bone.

Without vitamin D only 10-15% of dietary calcium and 60% of phosphorous is absorbed [3,4,5]. Vitamin D increases efficiency of calcium absorption to 30-40% and phosphorous absorption to approximately 80% [3-5,7]. As per a study [4] maximum bone density achieved at 40 ng/ ml of 25- hydroxyl D in circulating blood .below 30 ng/ml or less there was a significant decrease in intestinal calcium absorption [7]. That was associated with increased parathyroid hormone [8-10]. Parathyroid hormone enhances the tubular re-absorption of calcium and stimulates the kidney to produce 1,25- dihydroxyvitamin D [3-5,11]. Apart from regulation of calcium and phosphorous and bone metabolism, the discovery that most tissues and cells of the body have vitamin D receptor, and that they can convert the 25- hydroxyl D to its active form 1,25-dihydroxyvitamin D has provided new insights into the function of vitamin D.

ITS role in decreasing the risk of many chronic illnesses including common cancers, autoimmune diseases, cardiovascular diseases, type 2 diabetes mellitus, multiple sclerosis has raised great interest among clinicians to vitamin D. As a result there is tremendous increase in laboratory requests for vitamin D estimation. At this juncture we really need to know, what is the best clinical indicator of vitamin D STATUS, its reliability, how to monitor VITAMIN D metabolites during supplementation of vitamin, and how to interpret these results.

II. Types of Vitamin D Metabolites

There are approximately 37 vitamin metabolites that have been isolated [12]. Vitamin D₃ is synthesized by UV radiation and dietary sources. Vitamin D₃ is produced from previtamin 7-dehydrocholesterol. 1) Vitamin D₂ is produced from ergosterol. Some studies have established that humans can also metabolise vitamin D₂ to 25- hydroxyl D₂ and 1,25-dihydroxy D₂ [13]. 2) Vitamin D₃ does not have any intrinsic biological activity. It is present as 25- hydroxy vitamin D₃ in the liver, and is biologically inactive. 25-hydroxy vitamin D₃ is the major form of vitamin D circulating in the blood compartment. Vitamin D is supplemented either in D₂ form or in D₃ form. Studies suggest that 25-hydroxyvitamin D₃ levels rise when D₃ is supplemented. Vitamin D₂ is not that useful in rising 25- (OH) D as is vitamin D₃. Vitamin D₃ is more effective than vitamin D₂. [14,15]. 3)the active form 25-OH D₃ is 1α,25-dihydroxy vitamin D₃ is produced in the kidney. (Kidney as an endocrine gland) acts as a hormone. There is no reliable method available to estimate 1,25-(OH)₂ D₃. 4) 24, 25-dihydroxy D₃ is also produced in the kidney, and is a hormone. It is a active form. These two hormones are transported through blood to distal target organs. There they are internalized by binding to nuclear receptors or both nuclear and plasma membrane receptors to generate appropriate biological responses [16].

Table.1 Types of vitamin D

Types of Vitamin D	Production in humans	Properties
Vitamin D ₃ (Precursor-7-dehydrocholesterol)	In Skin produced by UV rays	Biologically inactive itself. Does not bind to VDR. Also supplied by diet
25-hydroxy D ₃		
Vitamin D ₂ (Ergosterol)	Not produce in humans	Supplied through fortified food are D ₂ supplementation
1α, 25-dihydroxy D ₃	Produced in kidney by 1α, 25-hydroxylase	Biologically active form of vitamin D. It is a steroid hormone. It acts through VDR
24,25-dihydroxy D ₃	Also produced in the kidney	Also a steroid hormone

III. Commonly Used Methods

1) Competitive vitamin D binding assays

In this naturally occurring vitamin D binding protein is the ligand binder and titrated 25- hydroxyl D₃ is the tracer [17]. 25-hydroxy D is taken from serum either by extraction or separated from other lipids by chromatography. Loss of 25-hydroxy D (endogenous) is compensated by titrating with 25- OH D₃ as an internal standard[18]. Advantages are a) the method is equally specific for estimating 25-OH D₃ and 25-OH D₂, b) extraction and chromatography methods reduce non specific interferences from other serum constituents.

Disadvantage is that the procedure is time consuming, needs expensive equipment and disposal of waste poses environmental hazards.

2) Immunoassays

Antibody was raised against a synthetic vitamin D analogue coupled to bovine serum albumin. As the structure of 25-OH D₃ and 25-OH D₂ differ only in their side chains, antibody has equal affinity for both molecules[20].the latest immunoassay method uses ¹²⁵I tracer[19]. In 2004 first chemiluminescence assay was introduced by Diasorin corporation. In 2007 the methods precision and sensitivity were improved. Manufacturer claims that this technique displaces 25-OH D₃ from binding protein completely and consistently. Co-specificity for 25-OH D₃ and 25-OH D₂ is supported by DEQAS survey in 2008 [21], another study published in 2006[22] did not support the claim.

3) Immunodiagnostic systems (IDS)

First came out with a radioimmunoassay method. It uses the antibody which is said to have 75% cross reactivity with 25-hydroxy D₂. The other method is enzyme immunoassay. It uses the same antibody. Several studies have been published about the detection of 25-hydroxyl D₂ by these methods. Enzyme immunoassays give higher values of 25-hydroxy D₂ [23] than those of IDS and Diasorin. Some studies produced comparable results between the assays [21]. The reason for this variation in recovery may be matrix differences. Between complexes[24]. In 2009 IDS came out with a FDA approved chemiluminescence method on iSYS automated analyser. The method claims to be specific for 25-hydroxy D₃ and 25-hydroxy D₂. All these methods could not detect 3- epimer of 25-hydroxy D.

Later Roche has developed a chemiluminescence method for Elecsys-2010/e411 and E-170/Cobas e601 systems. This assay is useful only in detecting 25-hydroxy D₃. An acidic pH change inactivates the DBP and liberates the bound 25-hydroxy D₃. Disadvantage is that it is not suitable to monitor patients supplemented with 25-hydroxyl D₂. Roche uses LC-MS/MS reference method, and a monoclonal antibody.

4) Chromatographic assays

These are 1) HPLC with UV detection, 2) LC-MS/MS-Liquid chromatography mass spectrometry, usually tandem mass spectrometry has been referred as “gold standard” technique for 25-hydroxy D₃[25]. Both methods can separate individual 25-hydroxy D₃ and 25-hydroxy D₂. Some authors advise to report total value and others advise to give total 25-hydroxy D and percentage of 25-hydroxy D₂ [25,26]. Gas chromatography - Mass spectrometry can be regarded as original definitive method for 25-hydroxy D[27]. They are very time consuming, column size is very big, needs lot of space and are seldom used now.

Commercial HPLC and LC-MS/MS kits consisting of reagents, standards and controls are available from chromosystems with trilevel set of human serum based calibrators and controls. ESA has a 25-hydroxyl D method with single calibrator, which is not based on human serum.

IV. Factors Contributing To Variations In Vitamin D Estimations

These factors pose a challenge to obtain the true value of patient's vitamin D levels [28]. Interferences in patient samples can potentially affect the vitamin D values. Different assay methods can also attribute to different vitamin D values. Potentially harmful factors that could contribute to the variations in 25-hydroxy D values are,

1. Preanalytical

Preanalytical issues are concerned with sample stability. Vitamin D and its metabolites are unstable compounds, on exposure to UV light, they are rapidly degraded. 25-OH D and 1,25-dihydroxy vitamin D are extremely stable compounds, as they are bound to vitamin D binding protein (DBP). These samples are opaque to UV radiation. [25] and they can be stored for days at room temperature without any significant change in 25-OH D concentration[29,30]. Repeated freezing and thawing also has no significant effect on serum concentrations of 25-OH D[31]. Blood collection tubes-majority of laboratories collect blood either in anticoagulant or gel tubes. Study done by Lensmayer G.L et al [32] said that gels have adverse effects on some steroid assays, including HPLC methods for 25-OH D. Another study done by DEQAS [33], said immunoassays were not affected by gels, but about one third of HPLC and few LC-MS/MS users reported spuriously high results. EDTA mixed samples gave low values in immunoassay methods, but HPLC methods were unaffected. Serum from Sarstedt Monovette tubes were found to give significantly raised levels in LC-MS/MS assays. Hence blood for 25-OH D measurement is probably best collected into plain tubes without anticoagulants or gels.

2. Analytical

Analytical variations are mainly due to sample interference accuracy of 25-OH D depends on the blood concentrations of vitamin D binding protein [34]. Variations also occur due to the presence of the human anti animal antibodies (HIAA) [35].

Assay characteristics

Among assay characteristics influencing the result are, the first problem which will change the assay is the lipophilic nature of the molecule makes the assay prove to interference from other lipids, like lipoproteins (matrix effects) extraction assays such as competitive protein binding assays are effected by matrix-effects [17]. But as matrix effects are unpredictable, they may vary from sample to sample [36,37]. There are always method related differences in individual samples. A common problem with non-extraction assays is the analyte has to be completely displaced from its binding protein completeness of vitamin D dissociation from DBP will determine the assay.

The second problem common to extraction and non-extraction assay is the preparation of standards. Stock standards are calibrated at A_{Max} i.e. 265nm. Purity of the standard is checked by calculating the ratio A_{Max}: A_{Min} which should be 1.5. If the ratio is lower than 1.5, the standard should be repurified (or) replaced [38].

Commercial working standards are often described as human serum based. To serum stock standard is added. From the serum endogenous 25-OH D is removed by activated charcoal at acidic pH. Both of them create matrix effects which brings variations in assays [24].

The third problem is invitro addition of 25-hydroxy vitamin D results in erroneous values by non-extraction assays. There is incomplete disruption of vitamin D binding protein from 25-hydroxy vitamin D. Almost all methods face standardization problems, because different predicates used by different manufacturers and lack of standard reference procedure and reference materials.

To improve the situation one study [22] proposed to develop-in-house reference method to provide an accuracy based for other testing methods. To take the assay uncertainty into consideration, while setting up vitamin D cut of value among different assays [24].

Post analytical

Post analytical factors are mostly to do with interpretation factors, and is discussed in section VII.

V. Selection Of Appropriate Method

Method choice depends on several factors 1) Sample throughput 2) Expertise of staff 3) Sample origin. i.e. whether to estimate 25-hydroxy vitamin D₂ (or) 25-hydroxy vitamin D₃ one has to look into which vitamin is being supplemented i.e. 25-OH D₂ (or) 25-OH D₃. Confusion can arise when a patient supplemented with vitamin D₂ is monitored by a method to measure only 25-hydroxy vitamin D₃ [39]. Occasional neonatal samples for 3-epi-25-OH D can be sent to a specialist centre for analysis. When small numbers of laboratory request for 25-hydroxy vitamin D, a good choice of method would be either HPLC (or) LC-MS/MS. Where there are more laboratory requests a dedicated instrument for 25-hydroxy vitamin D estimation would be appropriate. For long term vitamin D studies, continuity of quality of an assay is required. Immunoassays are affected by occasional changes of antibody (or) reformulation of reagents. This would make interpretation of studies difficult, as it was by NHANES [40].

VI. Type Of Vitamin D To Be Estimated

Blood concentration of total 25-hydroxy vitamin D (sum of 25-hydroxy vitamin D₂ and 25-hydroxy vitamin D₃) is the established biomarker to determine patient's vitamin D status [41]. Vitamin D₃ is mainly produce in skin through sunlight exposure. Vitamin D₂ is found in certain types of natural foods and fortified foods. Both forms of vitamin D can be obtained from vitamin D supplements [41]. Both forms of vitamin D are metabolized to 25-OH D in the liver and further to 1,25-dihydroxy vitamin D in the kidney, and both forms exhibit the same biological activities.

To monitor the effects of supplements it is crucial that the 25-hydroxy vitamin D assay used recognized both forms of vitamin D to accurately reflect patient's vitamin D status. Many vitamin D experts recommend the used of vitamin D assays with equal detection of vitamin D₂ and D₃ (42).

VII. Interpretation Of Vitamin D Results

Each laboratory provides reference interval to interpret results. These reference intervals vary based on based on local population, type of analytical method used. The reference ranges usually include 95% of the results of the population. For vitamin D obtaining reference intervals from local population does not work as vitamin D deficiency is worldwide[43]. Hence majority of such population derived reference ranges will be

low, which leads to under diagnosis of vitamin D deficiency. One such large population based study [44] gave 25- OH D range as 25-137 nmol /L. Other study [45] suggest a reference range of 100- 175 nmol/L. Several studies have been done [2-5,11] to confirm the presence vitamin D receptors in brain, prostate, breast and colon tissues, and also immune cells. These tissues respond to 1,25- dihydroxy vitamin D, the active form of vitamin D. Some of these tissues and cells express the enzyme 25-hydroxy vitamin D-1 α -hydroxylase[2-4,11]. 1, 25- dihydroxy vitamin D controls more than 200 genes, that includes genes responsible for the regulation of cellular proliferation, differentiation, apoptosis, and angiogenesis[2,3,50]. Due to these varied actions of 1, 25- dihydroxy vitamin D ,defining reference ranges for 25 OH D in each group of patients , might assist the clinician to supplement vitamin D in appropriate doses, to prevent vitamin D toxicity. Standing committee of European Doctors(CPME)[46] concluded that several diseases including colon cancer, infections, multiple sclerosis, type 1 and 2 diabetes are more prevalent in subjects with 25-OH D levels below 50 nmol/L.

Several studies have proved that results given by different methods are not comparable [32,37,47] making the interpretation all the more difficult. Survey conducted by DEQAS showed most major methods give results within about 10% of the consensus mean. Two possible solutions to these problems have been proposed [48], though entirely not satisfactory - 1) an arithmetical correction to be made to LC-MS/MS positive biased results. But the use of single correction factor for all results would be inappropriate. 2) Other Diasorin based radioimmunoassay should be adjusted to correct their negative bias.

This variability of results among laboratories using the same method, the reference range problem can be adjusted based on published method comparisons (eg. DEQAS).This should satisfy clinicians, who would wish to know whether their patient should be given vitamin D supplementation or not. There are also interpretation problems when a lab changes the method for vitamin D estimation. If the lab converts assay LC-MS/MS to fully automated 25-OH D immunoassay, there should not be a major change in the total 25-OH D value, as the previous LC-MS/MS also detected both vitamin D₂ and vitamin D₃.

The change comes only when converting from another immunoassay with partial D₂ detection. When no D₂ supplements are taken, there will not be much changes in the total 25- hydroxy vitamin D by fully automated method, which reflects the true vitamin D levels.

Table.2 Circulating concentrations of 25-hydroxyvitamin D [25(OH)D]

Serum 25(OH)D range2	Vitamin D nutritional status	Reference
>75 nmol/L (>30 ng/mL)	Sufficiency	[52]
>50 nmol/L (>20 ng/mL)	Sufficiency	[53,54]
30–50 nmol/L (12–20 ng/mL)	Insufficiency	[55]
12–30 nmol/L (5–12 ng/mL)	Deficiency	[53]
<12 nmol/L (<5 ng/mL)2	Severe deficiency	[53]

Table.3 Vitamin D Status in Relation to 25(OH)-D Levels in children’s[56]

Vitamin D Status	25(OH)-D Level, nmol/L (ng/mL)
Severe deficiency	≤12.5 (5)
Deficiency	≤37.5(15)
Insufficiency	37.5-50.0(15-20)
Sufficiency	50-250(20-100)a
Excess	>250(100)b
Intoxication	>375(150)104

VIII. Standardisation And External Quality Assessment Of Vitamin D

The main reason for variations in vitamin D values reported by different methods and different laboratories is variations in their standards. This stresses upon the need to standardize 25- hydroxy vitamin D assays to provide accurate measurement and reliable patient care. One such organization is DEQAS in U.K. It distributes serum samples to participants at a quarterly basis and statistically analyses submitted results to generate all lab trimmed means(ALTm)[51] To which each participant can compare their own method (www.deqas.org).With this measure interlaboratory imprecision (%CV) is reduced from >30% in 1995 to 15% in 2011,although the ideal %CV should be <10%.therefore there is a need to develop reference materials and a reference measurement procedure against which both chromatographic and immunoassays could be standardized. In 2009 National Institute of Standards and Technology (NIST) introduced reference materials SRM 972[49]. These reference materials helped to reduce variations in DEQAS samples.

These reference materials are spiked with exogenous metabolites (or) diluted with equine serum, the sample matrix is not suitable for many immunoassays [49]. Search for new reference materials that suit both LC-MS/MS and immunoassays is on.

A reference LC-MS/MS procedure is developed by NIST and university of Ghent .Clinical serum samples are available for labs to calibrate their current assay against the reference methods. Quarterly samples

for EQAS can be used. The goal should be bias $\pm 5\%$ and imprecision $\leq 10\%$ respectively. (www.cdc.gov/labstandards/).

IX. Conclusions

- 1) The knowledge of vitamin D₃ as a vitamin and as a hormone are very essential to assess the importance of 1 α , 25-dihydroxy D₃ in bone metabolism as well as in the metabolism of extra skeletal tissues through VDR.
- 2) The sources of vitamin D₃ emphasizes on exposure to UV rays for its synthesis. At the same time over exposure to UV rays can cause melanomas, thus the supplementation of vitamin D₃ or D₂ either as fortified foods or as medication gains importance. At this onset vitamin D₃ estimation becomes the centre point for monitoring vitamin D₃ levels or to diagnose the deficiency of vitamin D₃ in various diseases.
- 3) The estimation of vitamin D₃ is effected by several factors such as pre-analytical which mainly a concerned with sample stability and sample interference. The analytical variations depend on the concentration of vitamin D binding protein, and how vitamin D₃ can be dislodged from its binding protein. Apart from this vitamin D₃ results also show variations due to difference in internal standards, matrix effects, lack of reference methods and low availability of EQAS for vitamin D.
- 4) The best method for estimation of vitamin D₃ is the method which can estimate both vitamin D₂ levels and vitamin D₃ levels separately. There is still a lot of scope to research into newer methods and finding a way for uniform vitamin D₃ values between laboratories.
- 5) The interpretation of vitamin D₃ might need different cut off values when it comes to the vitamin D deficiency in extra skeletal tissues. Such as cancers autoimmune disorders, diabetes, infections etc. This provides a wide scope for research into vitamin D cut off ranges for each disease.

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