



# Vitamin D assays in clinical laboratory: Past, present and future challenges



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## ABSTRACT

Vitamin D status is usually assessed by measuring the serum 25-hydroxyvitamin D (25(OH)D) concentration. There has been a dramatic increase in 25-OHD requests over recent years prompting many laboratories to consider the use of automated immunoassays. In this presentation, we will discuss and compare the two major techniques that are used for measuring of vitamin D (the binding assay and chemical assay techniques).

Chemiluminescence immunoassays (CLIA), radioimmunoassay (RIA), and binding protein assay are belonging to the binding assay, while the chemical assay includes high performance liquid chromatography (HPLC) and liquid chromatography-tandem mass spectrometry (LC-MS/MS). Significant differences in the 25(OH)D determination were observed between various assays. Standardization and harmonization of 25(OH)D measurements are therefore urgently needed. The widespread introduction of well standardized assays in clinical laboratories is the challenge in the next years.

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## 1. Dramatic increase in vitamin D testing

Rickets was first described in the 17th century as outbreak in England. Following that Vitamin D was recognized as a very important component of the diet related to development of rickets and other bone diseases. Presently, rickets has been almost eradicated from most developed countries; however is still a very common problem in areas of the world where food is scarce.

The recent dramatic increase in vitamin D testing is primarily due to two causes: First, there has been a marked increase in vitamin D deficiency throughout the world. The second reason for that increase is the use of vitamin D as general health marker and the link between vitamin D deficiency and several diseases.

## 2. Metabolism

What we commonly refer to as vitamin D actually comes in two different forms: vitamin D2 and vitamin D3. Vitamin D2 is also known as ergocalciferol or calciferol. Vitamin D3 is also known as cholecalciferol (it derives from cholesterol). There are two main ways vitamin D gets into the body: through the skin and through diet. In the intestine, either dietary vitamin D is absorbed and trapped in the chylomicron molecules. In the skin, under the effect

of UV rays of sunlight, 7 dehydrocholesterol is converted to cholecalciferol (Vitamin D3). Vitamin D from the two sources is subjected to hydroxylation in the liver to form 25 OH Vitamin D. The hydroxylated vitamin D then gets alpha hydroxylation in the kidney to form 1,25(OH)<sub>2</sub> vitamin D, the active form of vitamin D. 1,25 (OH)<sub>2</sub> vitamin D increases calcium and phosphorous absorption in the intestine. It also interacts with the parathyroid gland to feedback in production of parathyroid hormone, therefore acting as a regulator of new bone formation. Vitamin D also being recognized as a very important player in signal transduction mechanisms in several organs like: brain, prostate, breast and colon tissue as well as immune cells. These organs have a vitamin D receptor and respond to 1, 25 (OH)<sub>2</sub> vitamin D.

In circulation Vitamin D is transported by Vitamin D binding protein, which belongs to albumin and alpha fetoprotein gene family. The concentration of vitamin D binding protein in plasma greatly exceeds that of 25 OH D (9 versus 50 n M) with less than 5% of available binding sites are occupied [1].

## 3. Measurement of 25 OHD

The analytical measurement of vitamin D is performed for two major reasons: to determine the nutritional status of vitamin D, and to monitor its therapeutic level. As mentioned before, there are two different types of vitamin D. To adequately monitor therapy, we need to be aware of what entity is the one measured in the

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different assays. If an immunoassay or protein binding assay is to be used, is the antibody reacting equally with both types of vitamin D? If the intention of measuring vitamin D is to monitor vitamin D2 therapy, then the assay must measure vitamin D2. The assays currently available in the market (US and EU) can be classified into binding and chemical assays. The binding assays are affected by matrix effects due to the tight binding of the vitamin D binding protein to vitamin D. Currently, automated immunoassays are very popular and practical for the clinical laboratory. Chemical assays have been originally more technically involved but are also now able to accommodate large number of tests/day. Current methods to measure vitamin D are RIA, HPLC, LC-tandem mass spectrometry and more recently CLIA. The specificity and accuracy of these methods are very variable. Two of them (RIA and CLIA) are immunoassay in which accuracy of the method will depend on the specificity of the antibody used (how well the antibody recognizes D2 and D3). The chemical methods (HPLC and LC-MS/MS) can report D2 and D3 independently.

The first automated vitamin D assay was based on Competitive-Protein Binding Assays (CPBA) for Nicholis Advantage analyzer [3]. It has the advantages of being inexpensive, can be performed on small sample size and Co-specific for 25(OH) D2 and 25(OH) D3. This assay underestimated 25-OHD at low levels and overestimated it at high levels [5]. Immunoassay methods were first reported in 1980s with a radioimmunoassay (RIA). This assay formed the basis for a subsequent chemiluminescent detection-based system. Radioimmunoassay (RIA): requires small sample size and incorporation of  $I^{125}$  as a tracer. It is not subjected to nonspecific interference, in addition to being rapid, inexpensive and accurate. Still requires the use of radionuclides and some RIAs discriminate between 25(OH) D2 and 25(OH) D3 [5].

Ultraviolet quantitation following HPLC is very stable and repeatable, provides separate quantitation of 25(OH)D2 and 25(OH)D3. But larger sample size is required, needs preparation step before chromatography and sometimes assay is subject to interferences with other compounds measured in the ultraviolet spectrum. High level of technical expertise is required.

LC-MS/MS has been referred as (Gold Standard) technique for 25-OH D3 [4] although result can be also erroneous. This technique requires the skills of an experienced analyst. Another caveat with LC-MS/MS is the presence of the 25(OH) vitamin D2 and D3 C3 epimers in pediatric specimens. If the assay is not optimized, vitamin D2/D3 result may be higher than expected in the pediatric population due to this epimer. Another publication, have shown that the C3 epimer may be present in adults as well [8].

#### 4. Standardization and external quality control assessment

With the availability of many vitamin D assays, differences in the reported 25(OH) vitamin D values for the same samples were observed among different assays.

These differences could impact the classification of patients' vitamin D status and so affect the clinical management of some patients. Is it appropriate to have a clinical decision limit without assay standardization? In 2010, the National Institute of Standards established a vitamin D quality assurance program in collaboration with the NIH. Data submitted by participants using the methodology of their choice is compared with the NIST standard (LC-MS/MS). CAP also provides proficiency testing materials.

CDC has introduced a Vitamin D Standardization-Certification Program to ensure reliable clinical vitamin D measurement. All assay manufacturers should participate in the CDC's

Standardization-Certification Program. This is especially important for the manufacturers in-house reference method and for assay measurement systems as they are being developed. The primary steps to standardization are as follows: [1] develop a reference system; [2] establish metrological traceability; and [3] verify "end-user" test performance [2]. When participants pass four consecutive surveys; they are awarded certification for 1 year. Renewal is an annual process.

#### 5. Controversies regarding Vitamin D testing

Past: over the past decade, a big number of studies linking low vitamin D levels to cancer, heart disease, diabetes, and other diseases led many doctors to routinely test vitamin D levels for their healthy patients. Consequently, laboratory professionals are confronted with the dual challenge of increasing testing volumes and helping clinicians navigate the complexities of vitamin D assays.

The current evidence suggests that the main beneficial effects of vitamin D supplementation relate to musculoskeletal, rather than extraskelatal. Moreover, of the exponential increase in vitamin D testing and supplement used in the past few years, has raised justifiable concerns that many vitamin D measurements are being undertaken without evidence-supported indications and many individuals are being supplemented with little evidence for benefit [7].

Present: In response to these concerns in 2013 [6], the Royal College of Pathologists of Australasia (RCPA) published a position statement to clarify the role of vitamin D testing in the context of vitamin D deficiency, with guidelines about who should be tested and when repeat testing should be performed. Also U.S. Preventive Services Task Force (USPSTF) published a new recommendation in November 2014 stated that, there's no practical reason for most people to get a vitamin D test. But there are exceptions.

People who might need testing include those who: have osteoporosis or other bone-related problems, have conditions that affect fat absorption, including celiac disease or weight-loss surgery or who are taking medications that interfere with vitamin D activity, including anticonvulsants and glucocorticoids.

Future: assay challenges include; move to SPE to allow improved sample cleanup, minimize extraction steps to ones that can be automated and trying to generate less waste.

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