



Review Article

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Vitamin D Assay in Human Serum Samples: A Review of Analysis Methods

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Abstract

Vitamin D was discovered about one century ago, which is part of the family of hormones. Vitamin D supplements have been commonly used in medical prescription. Deficiency of Vitamin D₃ has been related to various severe diseases, including cardiovascular disease, cancer and diabetes. Therefore, fast and reliable analytic methods for Vitamin D are very important for clinical diagnosis and treatment, as well as for biomedical research. To date, immunoassay, LC-MS/MS and HPLC are three major methods for the qualification and quantitation of Vitamin D. In this paper, we will go through the advantage, disadvantage and limits of each method and provide advice for future analysis of this group of hormone.

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Introduction

Vitamin D was discovered in cod liver oil by McCollum et al. in 1922. The active substance in cod liver for the treatment of rickets was found to be Vitamin D, which was a break-through discovery in the history of medicine (DeLuca, 2004). Modern research studies have shown that Vitamin D can be produced in skin by irradiation of sunlight (Chick and Roscoe, 1926). The photolytic synthesis produces Vitamin D₃ from a derivative of cholesterol (DeLuca, 2004). Vitamin D₂ can be produced by irradiation of ergosterol (DeLuca, 2004). Unlike other vitamins, Vitamin D is part of the family of hormones.

Generally, it is not present in common foods, either vegetation or meat with exceptions of some very uncommon food such as fish liver oils (Ross et al., 2011).

The structure of Vitamin D₂ was determined by Askew et al. in 1931, while the structure of Vitamin D₃ was unveiled by Windaus et al. in 1936. The structure of the Vitamin D family is shown in Fig. 1 (Wang et al., 2016; Wolf, 2004). Vitamin D is converted in the human body to an active form and circulated as 25-hydroxyvitamin D [25(OH)D]. The metabolism is shown in Fig. 2 (DeLuca, 2004). In the application of current clinical practices, the level of

25(OH)D in serum is monitored. There are three goals to achieve by measuring 25(OH)D in clinical practices (Holick et al., 2011). The first is to diagnose Vitamin D insufficiency or deficiency in patients (Holick, 2007). Secondly, it is important to monitor the response to Vitamin D supplements (Zeghoud et al., 1997). Thirdly, it is also necessary to diagnose Vitamin D toxicity in some rare cases (Pettifor et al., 1995).

The physiological role of Vitamin D is closely linked to the mineralization of bone, the regulation of calcium ion and phosphate concentration in the bone and plasma, and a number of cancer prevention mechanisms (Fig. 3) (Wan et al., 2016).

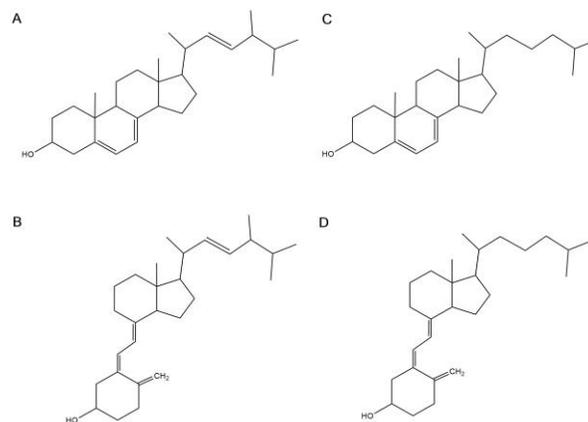


Fig. 1: Structure of ergosterol (A), calciferol or vitamin D₂ (B), 7-dehydrocholesterol (C), and cholecalciferol or vitamin D₃ (D).

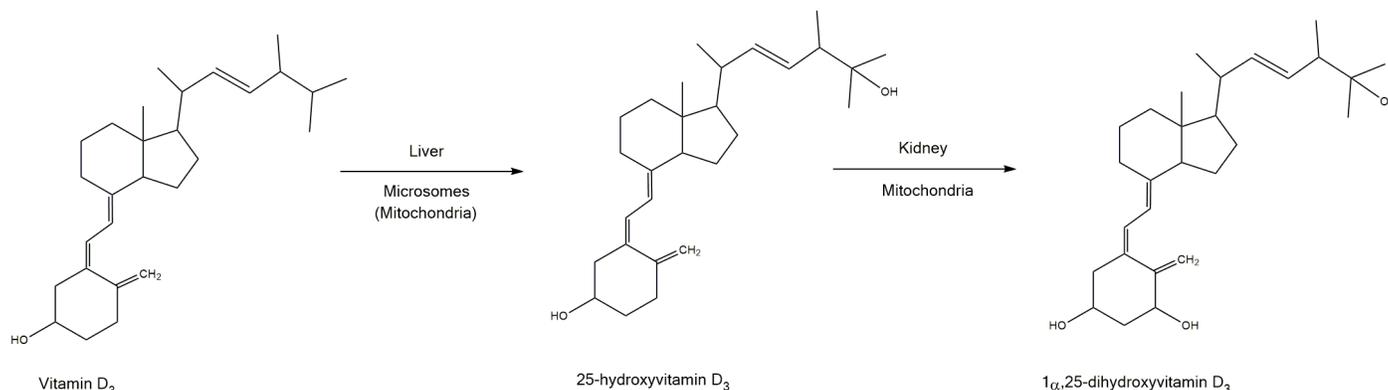


Fig. 2: Vitamin D₃ conversion to 25-hydroxyvitamin D in the liver mitochondria by vitamin D 25-hydroxylase. In the kidney, 25-hydroxyvitamin D is further hydroxylated to form the 1,25-dihydroxyvitamin D by 1-alpha-hydroxylase (DeLuca, 2004).

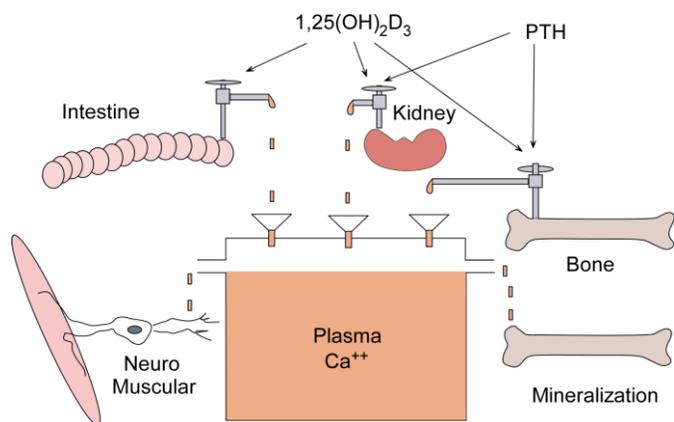


Fig. 3: Diagrammatic representation of the role of the vitamin D hormone and the parathyroid hormone (PTH) in increasing plasma calcium concentrations to prevent hypocalcemic tetany (neuromuscular) and to provide for mineralization of the skeleton.

The common test methods for the determination of Vitamin D level in serum can be divided into three

categories: immunoassay, LC-MS/MS, and HPLC. Immunoassay is widely accepted as a standard monitoring method used for most patients due to its simplicity and inexpensive operation. Immunoassay will generate accurate quantitative results of the total 25(OH)D level in serum samples.

LC-MS/MS is used in some special cases. It can be used to differentiate the two forms of Vitamin D in serum, which are 25(OH)D₂ and 25(OH)D₃. In infant patients, the amount of the C-3 epimer of 25(OH)D₃ is high in blood and it can be separated by LC-MS/MS to remove interference of the Vitamin D levels. Also, LC-MS/MS can be used to quantify the 1,25(OH)D level, which is used to assess the condition of patients on Vitamin D therapy with chronic kidney disease.

HPLC separation and quantitation of Vitamin D has been developed with UV detection. Three types of separation including normal phase, reversed phase, and a

combination of the two have been reported. The sample preparation and the interpretation of the chromatogram was complicated, which limited the use of HPLC methods in routine clinical works. However, some of the HPLC methods achieved great sensitivity and selectivity over other methods.

In this review, the three categories of Vitamin D analysis will be covered in detail with examples in the literature. The advantages and disadvantages of each will be carefully compared and discussed. The trend of current works and the guidance for future research will also be provided.

Immunoassay

Immunoassay, especially radioimmunoassay, was developed by Rosalyn Sussman Yalow, an American female medical physicist who won the 1977 Nobel Prize in Physiology or Medicine (Yalow, 1983; Glick, 2011). It has been a revolutionary technique in clinical testing of almost every field of medicine, opening doors to the measurement of hormone, vitamins, drug substances, biomarkers, and other macro-molecules.

The first work of clinically used immunoassay on the analysis of Vitamin D in blood samples was published in 1971 by Haddad and Chyu. The use of a simple procedure of competitive radioimmunoassay eliminated the lengthy and tedious preparation in the chemical separation and bioassay methods. In the commonly used competitive ligand radioimmunoassay, a certain amount of radioactive isotope marked 25(OH)D was added together with the unmarked sample, into a binding protein. If there is no 25(OH)D in the sample, the bonded form will only be the radio-marked ones.

The existence of 25(OH)D in the sample will compete with the marked and inhibit the binding of the radio-marked molecules. Thus, a detection of radioactive level of the resulting bonded material enables the calculation of the level of the 25(OH)D in the sample.

With the fast-growing demand for Vitamin D test worldwide, numerous commercially available test kits have been developed and adopted as a routine clinical standard, such as the products from Abbott (Architect), DiaSorin (LIAISON), IDS (ISYS), Roche (E170), and Siemens (Centaur). The Food and Drug Administration (FDA) has approved the radioiodine based method for clinical use. Many of them have achieved automated analysis to increase the output, reduce the work load, and eliminate possible human error. The operation of the radioimmunoassay needs minimal amount of training, and the test kits are available at relatively low prices. In addition, the processing time is fast. All those factors lead to a tremendous popularity. However, the reliability of the methods has been questioned.

Characteristics for some commercial Vitamin D assays are listed in Table 1. As a leading provider of commercial assays, DiaSorin claimed a wide linear range of 4-150 ng/mL, 100% recovery of 25(OH)D, and a good inter-assay precision of $\pm 11.7\%$. However, a lower recovery of 82% was reported by Carter et al. (2007); Hollis (2000) also pointed out that many of the commercial assays including the products from DiaSorin and Immuno-Diagnostic Systems (IDS) were not standardized against reference material. In the range of 10-40 ng/mL, the immunoassay could have underestimated the level of total circulating 25(OH)D (Hollis, 2000).

Table 1. Characteristics for common commercial Vitamin D assays.

Assay	Extraction	Range (ng/mL)	LOD (ng/mL)	Precision
DiaSorin (2016)	Acetonitrile	4-150	4.0	11.7%
IDS (2015)	NaOH /Acetonitrile	0-225	3.0	5.3%
Abbott (2016)	Ethanol	0-160	3.1	3.7%
Roche (2012)	Buffer /Acetonitrile	3-70	3.0	11.5%
Siemens (Chen et al., 2012)	N/A	4.2-150	4.2	11.9%

In a study by Turpeinen et al. (2003) the test kits from DiaSorin and IDS was closely compared with a validated HPLC assay. The results showed in the range of the claimed concentrations, the coefficient of variation (CV) are generally acceptable (between 4%

and 17%). However, the correlation between the HPLC and the DiaSorin or IDS methods are poor. This was also mentioned by Hollis et al. with a hypothesis of problems in the pre-treatment and/or extraction procedure (Hollis 2000).

LC-MS/MS

LC-MS/MS has used as reference methods for the validation of the immunoassay methods mentioned above. The LC-MS/MS carries great identification and quantitation capabilities. It was considered to be the “golden standard” for the identification and quantitation of many analytes. However, the competitiveness and higher knowledge requirements for the operators limited the use to the research purposes other than regular tests.

There are some LC-MS/MS methods developed for the quantitation of Vitamin D related metabolites. Most of the methods require the use of derivatization in the sample treatment. For example, in the study by Higashi and co-workers, the derivatization was performed using a Cookson-type reagent, which increased the complexity of the method (Higashi et al., 2000). In a published method by Saenger et al. (2006), deuterated Δ^9 -THC-D₃ was added as internal standard. The three major compounds of 25(OH)D₂, 25(OH)D₃ and Δ^9 -THC-D₃ were successfully separated using a simple HPLC procedure with methanol and acetate buffers. The run time was 6 minutes and all three compounds of interest were eluted within 3 minutes. Then the analytes were ionized using electrospray ionizer. The transitions used when monitoring the multiple-reaction were 318.15>196.20 for Δ^9 -THC-D₃, 401.15>365.25 for 25(OH)D₃, and 413.15>355.20 for 25(OH)D₂. The quantification was achieved by calculating of the integrated peak area ratio between the two Vitamin D derivatives and the internal standard Δ^9 -THC-D₃. The performance of this method was promising with a very low limit of detection at 0.09 ng/mL, a linear range of 0-200 ng/mL and an inter-assay CV of 11.5% (Saenger et al., 2006).

Another example of robust and reliable LC-MS/MS analysis of Vitamin D was reported by van den Ouweland et al. (2010). The performance of the developed LC-MS/MS method was compared to some popular commercial immunoassays including DiaSorin and Roche ECLIA. There are several LC-MS/MS methods published and the major difference is the procedure of calibration, which is also the major factor affecting the CV (Leino et al., 2008; Roth et al., 2008). In this study, the newly released reference standard material for 25-OH Vitamin D was used (SRM 972 and SRM 2972) (NIST, 2009 and 2014). The use of

common standard was likely to reduce the inter-laboratory CV for individual test methods.

In Ouweland's method, the sample was treated to remove the protein from the serum, followed by a solid-phase extraction. Strata C18-E SPE column was used and the recovery rate was calculated to be 85%. Then a gradient HPLC was used to separate the analyte of interest. The retention times are 3.01 min for 25(OH)D₃ and 3.06 min for 25(OH)D₂, with acceptable separation. The fragments of 25(OH)D₃ and 25(OH)D₂ was detected by selected reaction monitoring using m/z transitions of 401.5>159.2 and 413.4>83.1, respectively.

Limit of detection of the above method was 0.8ng/mL and the range was tested to be 0-220ng/mL, which provided a good coverage for common patients. When comparing with the immunoassay results, the DiaSorin kit showed a good correlation along the range. However, Roche ECLIA clearly overestimated the values of vitamin D in patients with higher level of vitamin D (above 20 ng/mL).

Despite of the complexity and high cost of equipment and operation, LC-MS/MS continuously delivered reliable data of vitamin D in human samples. With the commercialization of common standards by NIST, the LC-MS/MS has become a reference method for the performance evaluation of other assays.

HPLC

As a common analyte, Vitamin D assays using HPLC have been investigated and reviewed thoroughly in the late 1990s and early 2000s. There are numerous HPLC methods developed to separate Vitamin forms of 25(OH)D, 1,25(OH)D₂ and 24,25(OH)D₂ as well as many metabolites.

The first measurements using HPLC can be dated back to the late 1970s. Eisman and co-workers achieved 72.2% recovery of ³H-25(OH)D₃ with UV detection at 254 nm (Eisman et al., 1976). Internal standards whether prepared in house or available from NIST were both widely used in the quantification.

Conflict of interest statement

Authors declare that they have no conflict of interest.

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