VITAMIN D ESTIMATION: PROTOCOLS, CHALLENGES AND RECOMMENDATIONS

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ABSTRACT

Vitamin D, a pro-hormone, is not only important for bone health, but is also involved in other diseases such as multiple sclerosis, irritable bowel syndrome, type I diabetes, cardiovascular disorders and a variety of cancers. These findings have emphasized the need for determining vitamin D status in a convenient and cost-effective way. Measurement of vitamin D is not an easy task or straightforward procedure. There are many issues/challenges related to the testing procedure like different sources and metabolites, lack of harmonization between different methods and structural problems. In this context, present review highlights the importance of vitamin D determination in human health and diseases related to its deficiency. Problems associated with vitamin D measurements are also being described. Available methods of vitamin D determination were critically compared in order to gather logical suggestions for reliable and accurate determination. According to the reviewed literature in this regard, inexpensive and high output methods like Diasorin Liaison Total can be employed for routine use, however, low readings need to be repeated by LCMS, as the performance of Diasorin Liaison Total drops significantly with very low reading. In addition, more work should be done on standardization.

KEYWORDS: Vitamin D, LCMS, Diasorin Liaison Total

INTRODUCTION

Vitamin D is a pro-hormone which was discovered in 1922[1]. Vitamin D can be obtained from food sources as well as produced by human skin via sun exposure. In the past, vitamin D serum status was linked only to bone metabolism and bone related diseases. In contrast, since years ago, the importance of vitamin D in many other body functions has been discovered. For example, vitamin D was found to have a role in cell proliferation and body immunity[2]. In addition, relation between vitamin D deficiency and many diseases have been reported. For example, vitamin D deficiency was found to be associated with multiple sclerosis, irritable bowel syndrome, type I diabetes, cardiovascular diseases and various forms of cancers[3]. Moreover, researchers found that vitamin D deficiency may be linked with increased risk of myocardial dysfunction in type 2 diabetic patients. However, it was also suggested that vitamin D supplements might be useful in asthma and chronic obstructive pulmonary disease (COPD)[4,5]. At the same time, wide population spectrum has been diagnosed as vitamin D deficient. Due to its importance, vitamin D is being focused as vital research topic[6]. In parallel, laboratory requests for vitamin D estimation have been increasing because of its pronounced biological roles as well as associated deficiency diseases. Vitamin D testing has been recognized as routine. For example, according to CLN survey which was done in USA, more than 25% of labs reported that vitamin D requests increased by 100% or more between 2006 and 2008 while more than 50% increase was reported by half of labs at same period. In addition, MyoClinic lab reported that 61,000 tests per month were done in 2008 compared to 19,000 tests in 2006[7]. Moreover, 200% increase was reported by Aga Khan University clinical laboratory in Pakistan from 2005 to 2008[8]. The increase in annual requests of vitamin D was even more in Auckland, New Zealand, where four times increase was reported between 2000 and 2010[9]. By this explosion of vitamin D interests, proficiency of vitamin D testing has been a matter of clinicians and scientists' concern. For instance, participants in the International Vitamin D External Quality Assessment Scheme (DEQAS) were increased from 141 labs in 2001 to 670 labs in 2009[10]. Overall, high number, well performed tests are needed to be done in shorter time periods which is not an easy task. Testing of vitamin D faces many difficulties and challenges. In this review, information on some important aspects of vitamin D estimation and accompanied analytical challenges is being presented. Furthermore, details of few common methods are also discussed. Review of literature is concluded by providing recommendations for better testing practice.

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CHEMISTRY OF VITAMIN D

Vitamin D is a hydrophobic molecule with steroid like characters. It is found in two forms which are vitamin D2 known as ergocholecalciferol and vitamin D3 named cholecalciferol. Ergocholecalciferol is usually classified as plant form because it is obtained from plant food sources and supplement medicines. On the other hand, cholecalciferol can be either obtained from animal food and supplementations or produced by skin sun exposure. Vitamin D3 is known as animal form and sun exposure regarded as its important

source[11,12]. Vitamin D (D2 and D3) is biologically inactive. Vitamin D is metabolized in liver by hydroxylation and converted to 25(OH) D which is also an inactive metabolite. The 25-Hydroxylated vitamin D regarded as the body pool of vitamin D where it is the most common form in the body. Then, 25(OH)D is activated in the kidneys by additional hydroxylation step at position 1 to produce the active $1,25(OH)_2$ D form. This active form only exists for very short time. Additionally, $24,25\ (OH)_2$ D which has no biological importance is also formed at lesser extent[11,13]. Overall, vitamin D obtained from different sources and exists in different forms inside the body. In addition, both VitD2 and VitD3 are used in supplementation.

CHALLENGES OF VITAMIN D ANALYSIS

Different sources and metabolites

Presence of different forms or/and metabolites in addition to dual sources of vitamin D contribute in many analytical challenges. Two of these difficulties will be focused. Firstly, absence of clear cut off for insufficiency and recommended doses or recommended sun exposure time. Many other variations also contribute in this issue as seasonal variation, skin pigmentation and racial differences. In this regard there is a controversy about who can be categorized as insufficient, deficient, and optimal. However, person with serum level below 25nmol/l or 10ng/ml is classified as deficient while serum level 25 to 50nmol is classified as insufficient. Nevertheless, some studies nominate 75-87.5 nmol as recommended level[11,14]. The debate about optimal and sup-optimal ranges may result in different clinical classifications hence different clinical outcomes.

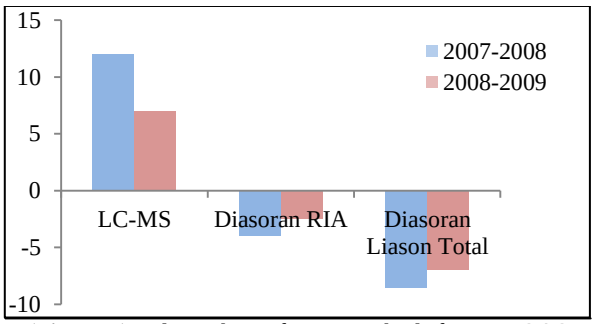
The other issue is defining which metabolites/form should be measured. Although 25(OH) D is the commonly used indicator for vitamin status, 25(OH) D2 and 25(OH) D3 separately or as a whole is still a debatable point. On one hand, Vitamin D2 is also activated and commonly used in many countries as supplement. Therefore reporting of only D3 is regarded as an incomplete picture. On the other hand, vitamin D2 has only one third activities compared to vitamin D3 and reporting them as one analyte could be misleading[15]. In addition, these forms cross react and form specificity issue in many testing principles. According to CLN survey, most of labs report total vitamin D[7]. This is not regarded a problem in normal situations as the concentration of vitamin D2 is quite low in compare to D3. Nevertheless, this point needs to be considered in many cases such as people use vitamin D2 as supplement. Theoretically reporting of each form separately seems to be the proper way especially many available methods measure fractionated D2 and D3. However, the interpretation problems and reference ranges are still the important limitations.

Lack of harmonization

In addition to the controversial reference ranges, there is some lack of agreement between labs, this was reported by many programs and clinical societies like vitamin D metabolites quality assurance program which was established in USA by the National Institute of Standards and Technology (NIS) in collaboration with the National Institutes of Health Office[16].

The use of different method could be the main cause. For instance, High Performance Liquid Chromatography (HPLC) gives higher results than Radio Immunoassay RIA and ELSIA for $25(OH)D_2$ while the opposite for $25(OH)D_3$[17]. These variations may result in different clinical classifications also between labs use same cut offs or even within the same lab that uses two different methods[18]. This problem is a recognized one and not uncommon. For example, in UMass Memorial Medical Center, by testing vitamin D for group of people by immunoassay and by Liquid Chromatography Mass Spectrum (LCMS) , more than two third of tested individuals classified as insufficient by immunoassay while less than one third in the case of LCMS[7].

These variations have been narrowing and better agreements are being achieved due to many factors. One of these factors is the International Vitamin D External Quality Assessment Scheme (DEQAS) which was implemented in 1989. This quality assurance program was founded as a response of poor vitamin D proficiency reported in many studies. Since that date, the participants increase while coefficient of variation between them declines. A 17% decrease in inter-laboratories imprecision in 15 years-time (1994-2009) was reported[10,19]. However, CV is still 15.3%, which does not meet the needed CV according to experts' opinion that states 10% as target coefficient of variation (CV) for routine measurements[20]. In addition, deciding which one of varied methods is more precise sill undoable because of absence of a certain gold standard protocol to which other methods can be compared and lack of standardization[10]. Some differences are shown in (figure 1) below.



(Figure 1) Relative bias of some methods from DEQAS calculated mean According to data obtained from two different cycles[10]

Structure related problems

Vitamin D is a hydrophobic molecule for this reason it needs to bind to a carrier protein in order to be transported as 85% of vitamin D attaches to vitamin D binding protein and the other portion to albumin. There is a very small free amount. 25(OH)D form

which usually is measured while the other portion is strongly binds to the binding protein.

This property adds some challenges. For example, in many used protocols, vitamin D needs to be extracted. In this aspect, extraction of vitamin D is not challenge free process because it is very stable in serum and labile in other fluids. In addition, it is insoluble in aquatic phases. Therefore, extraction in organic phases is needed which may need some drying steps. However, some methods which do not need an extracted sample have been developed but their results might be affected by the used agents which is known as matrix effect[21,22].

In addition to the above structural issues, the variability in the percentage of free 25(OH) D levels in compare to total vitamin D among different clinical population was also highlighted in some studies and it might have a clinical significance[23]. Moreover, the orientation change of hydroxyl group at carbon 3 of the steroid nucleus was reported and its biological importance has been questionable[24].

CURRENTLY USED METHODS

Historical background

First vitamin D assay was designed on protein binding competition in 1970. Then HPLC was founded in 1978. Later, in 1985 Radio immunoassay (RIA) have been commercially available. Then other methods were introduced like Enzyme Immunoassay (EIA), Automated RIA, and mass spectrum[8].

Immunoassays

There are manual and automated available immunoassays and most of them are competitive. Manual immunoassays usually have simple procedures which are easy to follow. But the long incubation time, low throughput specificity issues and inappropriate extraction techniques are important limitations. Manual Radioimmunoassay (RIA) like the one designed in 1985 by the diagnostic company Diasorin is a very common used example of this type. However, some other manual immunoassays do not need an extraction step such as Direct EIA. In this procedures turnaround time can be diminished, also simpler and inexpensive procedure is applied. Nevertheless, matrix effect is the main problem in addition to low throughput[25,26].

On the other hand, automated procedures offer important advantages. For example, Diasorin company developed in 2004 a fully automated Chemiluminescent Immunoassay (CLIA) named Liaison Total, this method is widely used nowadays due to the many offered advantages. It is an extraction free method that can run a large number of tests and gives the results in about 65 minutes. These benefits are important to cope with the tremendous increase in the request numbers. Diasorin Liaison Total is also technically simple. However, matrix effect is still a limitation. In addition, low sensitivity is another issue faced by this technique. Evidently, according to RCPAQAP program-cycle number 33 lower limit of detection for this device is 33 nmol/ml[26,27,28,29,30].

Physical detection methods

The more common non-immunological principles which are used in vitamin D testing are HPLC and LCMS. HPLC method has very good performance and it can be automated. In addition, HPLC is high sensitive with low detection limit. However, there are some limitations like low throughput and large sample size. Moreover, skillful operator is needed[30,31,32].

Liquid Chromatography Mass Spectrum LCMS is another sensitive method which is regarded as a golden standard in some practices. Matrix effect is minimized and standardization can be controlled by the operator. Moreover, LCMS automation is also available which help to alleviate the operator's effect. In the other side, low throughput, high establishment cost and needing of skilled operator are the main problems faced by technique. In addition, positive bias compared to many other methods is documented[7,27,30,31].

Suggestions for better practice

For current practice, method like Diasorin Liaison Total can be used for routine use as it offers inexpensive, high number of tests and good performance except for too low readings. In parallel, lower readings can be repeated on LCMS especially vitamin D tests are usually not urgent.

For future work, although the agreement between used methods is now increasing, lack of standardization is the most issue in vitamin D testing practice. Likewise, work in standardization is the most urgent. In addition, national compulsory proficiency programs need to be implemented and new programs also need to cooperate with DEQAP which is an international and non-mandatory program. The promising picture that supports that is the decline in CV since DEQAP has been implemented.

CONCLUSION

Discovered biological roles of vitamin D drastically increased number of the requested lab tests to check vitamin D status. Measuring of vitamin D is not an easy task or straightforward procedure. This is because of many issues faced by this testing process. Although some improvement like CV decline and closer agreements between methods have been achieved in the last years, different metabolites and sources, structural nature and lack of standardization still exist as important issues. In the future, more proficiency testing programs need to be implemented. Moreover, working cooperatively between implemented procedures on standardization issue should be the priority.

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