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Review article
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CHALLENGES IN VITAMIN D ANALYSIS

IZAZOVI U ANALIZI VITAMINA D

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Summary: Vitamin D is an important determinant for the regulation of calcium and phosphorus levels and mineralization of the bone. The most reliable indicator of vitamin D status is the measurement of plasma or serum 25OH-D concentration. Several studies reported discrepancies between the results of assays. These high variabilities in 25OH-D measurements are due to used assay technologies and lack of standardization against the reference materials. Different assays have been employed for the measurement of 25OH-D levels: Competitive Protein Binding Assays, immunoassays, direct detection methods. Choosing an assay platform is important both for clinical laboratory professionals and researchers, and several factors affect this process. Recently, liquid chromatography and tandem mass spectrometry is an alternative method to traditional assays and provides higher specificity and sensitivity than many assays; therefore, it has been suggested as a candidate reference method for circulating 25OH-D3. Standardization of methods for the quantification of 25OH-D by using the human-based samples would reduce the inter-method variability. The best way for laboratories to demonstrate the accuracy of their results is by participating in the external quality assessment scheme. Standardization of the assays is also required to provide clinicians with the accurate tools to diagnose hypovitaminosis. In addition, assay-specific decision limits are needed to define appropriate thresholds of treatment.

Keywords: 25 Hydroxy Vitamin D, assay, standardization, external quality control, LC-MSMS

Kratak sadr`aj: Vitamin D je važna determinanta u regulaciji nivoa kalcijuma i fosfora i mineralizaciji kostiju. Najpouzdaniji indikator statusa vitamina D je određivanje koncentracije 25OH-D u plazmi ili serumu. Nekoliko studija je pokazalo neslaganja između rezultata različitih testova. Ovako velike varijacije u određivanjima 25OH-D su posledica korišćenih tehnologija testova i nepostojanja standardizacije u odnosu na referentne materijale. Različiti testovi se koriste za određivanje koncentracija 25OH-D: testovi kompetitivnog vezivanja za proteine, imunoodređivanja, metode direktnog određivanja. Izbor platforme određivanja je važan i za kliničko-laboratorijsku praksu i za istraživanja i nekoliko faktora utiče na ovaj proces. Odnedavno, tečna hromatografija i tandem masena spektrometrija predstavlja alternativu tradicionalnim testovima, ima veću osetljivost i specifičnost od mnogih testova i predložena je za kandidata za referentnu metodu određivanja 25OH-D3. Standardizacija metoda za kvantifikaciju 25OH-D upotrebom uzoraka humanog seruma bi smanjila varijacije između metoda. Najbolji način za potvrdu tačnosti određivanja rezultata u laboratoriji je učešće u programu spoljašnje kontrole. Standardizacija testova je takođe potrebna da bi se kliničarima obezbedile tačne informacije za dijagnozu hipovitaminoze. Takođe, neophodni su nivoi odluke specifični za test da bi se definisale odgovarajuće vrednosti praga za terapiju.

Klju~ne re~i: 25 hidroksi vitamin D, test, standardizacija, spoljašnja kontrola kvaliteta, LC-MSMS

Introduction

Vitamin D is a pro-hormone, known for its important role in the regulation of calcium and phosphorus levels and mineralization of the bone. Hypovitaminosis D is known to contribute to osteoporosis through decreased calcium absorption, subsequent secondary hyperparathyroidism and increased bone resorption. For this reason, decreased vitamin D levels are usually associated with the increased parathyroid hormone (PTH) levels. Recent studies have shown that the Vitamin D receptors are present in a variety

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of cells and have biological effects which are far beyond the mineral metabolism (1). Low Vitamin D levels have been found to be associated with the asthma in children (2), endothelial dysfunction (3, 4), harmful immunomodulatory effects (5), cardiovascular risk (6), cognitive impairment (7), and lost antitumoral activity potentiating a number of cytotoxic anti-cancer agents (8). In oncology patients, it has been shown that low serum vitamin D levels predict an advanced stage of disease (9).

It has been estimated that globally more than one billion people are vitamin D deficient and in the States, more than 75% of the adult population is vitamin D insufficient (1, 10). Increases in vitamin D testing is attributed to growing global deficiency due to blockage in sun exposure and increased number of evidence between vitamin D deficiency and health conditions.

Vitamin D is metabolized in the liver to produce 25-OH-Vitamin D (25OH-D) and 1,25 (OH)2- Vitamin D is produced in kidneys (11). 25OH-D is a predominant form in the circulation and generally accepted as the best single marker of vitamin D status (1, 12). There are two types of 25OH-D found in the circulation: 25-OH-Vitamin D2 (25OH-D2) is also called ergocalciferol and derives mainly from plants and fish (13). 25-OH-Vitamin D3 (25OH-D3) or cholecalciferol accounts for approximately 95% of the circulating 25OH-D pool, whereas 25OH-D2 represents only a minor fraction unless vitamin D2-containing medication is taken by the individual (13).

1,25 (OH)2-Vitamin D is closely regulated by PTH and intestinal calcium. It circulates at extremely low concentrations what makes it more difficult to be measured accurately. Since vitamin D itself is tightly bound to vitamin D binding protein, it is the most highly lipid soluble form of the vitamin D (14).

25OH-D is better indicator of the patient's vitamin D status than the vitamin itself. This is because the hydroxyl group makes 25OH-D less fat soluble and makes it have lower affinity to vitamin D binding protein than the actual vitamin. These factors make the circulating concentrations of 25OH-D about 1,000 times more concentrated than the steroid hormone form of vitamin D. 25OH-D levels also correlate well with the clinical signs of vitamin D deficiency (15).

Measurement of 25-Hydroxyvitamin D

Competitive Protein Binding Assays

The history of developing a sensitive method for the estimation of 25OH-D levels dated back to nearly half a century. At first, the 25OH-D methods were categorized into two types: 1- Physicochemical methods and 2- Biological assays (16). The early gas chromatographic analysis of vitamin D was developed by Kodicek and Lawson in 1967 and by Sheppard et al in 1972 (17, 18). Edelstein et al. (19) argued that no suitable physicochemical methods have been available to estimate the amounts of vitamin D levels in animal tissues. Furthermore, these methods were time-consuming in separation procedures for the elimination of the interfering compounds with similar chemical properties, such as retinol or cholesterol. For this reason, biological assays gained importance to be used routinely in laboratories for the analysis of both human materials and analysis of animal food stuff, fish oils and many pharmacological preparations (16). But it was observed that low sensitivity, the cost of the analysis, labour and time consumption were the main disadvantages of bioassays (16).

Characterization of binding proteins of vitamin D led to development of competitive protein binding (CPB) assays for vitamin D (19). In the first reported CPB assay, the estimation of cholecalciferol and its 25-hydroxy metabolite in plasma by using the specific vitamin D -binding protein from rat serum was described (20). In the assay, beta-lipoprotein isolated from the human plasma was used as a carrier for steroids to overcome limited solubility of the steroids in water. The main disadvantage was the time factor. Several days were required for equilibration and displacement. This factor prevented the routine usage of this method for the estimation of vitamin D and its 25-hydroxy metabolite.

Another CPB radioassay for 25-hydroxycholecalciferol was reported by Haddad and Chyu in 1971 (21). In this assay, specific binding protein isolated from the kidney and tritiated 25-OH vitamin D3 as a tracer was used. Addition of absolute ethanol into the assay system overcame the solubility problem. Reaching the equilibrium displacement in 60 minutes provided this method simple and sensitive for routine estimation of 25-hydroxycholecalciferol levels. This method estimated 25-hydroxycholecalciferol levels as low as 4 ng/mL. Advantage of this assay was its co-specificity for 25-OH vitamin D3 and 25-OH vitamin D2 levels, which made it suitable for monitoring of patients treated with ergocalciferol. Free steroid was separated from the bound steroid by using the charcoal coated with dextran (21).

Chromatographic methods using the silicic acid columns to separate 25-hydroxycholecalciferol from other vitamin D metabolites were found to interfere with CPB assay and produce erroneous blank values (16). These were the results of substances produced during chromatography, which derived from impurities in the silicic acid produced by the interaction of the solvents and the silicic acid (16). Edelstein et al. (22) developed CPB assay for 25-hydroxycholecalciferol, which eliminated the interfering substances by using small Sephadex LH-20 columns. It was reported that in spite of the difference in sensitivity and specificity of CPB and biological assays, high correla-

tions were found in patient samples (16). All these attempts confirmed that 25-hydroxylated metabolite was the main metabolite of vitamin D in the circulation (16). Further studies on development of similar CPB assays were not successful. An automated CPB method (the Nichols Advantage Analyser) was introduced in 2004, but was withdrawn in 2006 (23).

Immunoassays

The first 25OH-D radioimmunoassay was developed by Hollis and Napoli (24). The assay antibody was raised against a synthetic vitamin D analogue coupled to bovine serum albumin. This antibody was co-specific for 25-hydroxyvitamin D3 and 25-hydroxyvitamin D2 (25). The first version of the assay used tritiated 25OH-D, but the method was modified to incorporate an 125I tracer (26). The Hollis and Napoli assay was the basis of the first commercial 25OH-D kit, originally marketed by Incstar and currently by DiaSorin Corporation (Stillwater, MN) (24). 25OH-D is extracted by denaturing the vitamin D binding protein (DBP) with acetonitrile. Since it was the only RIA detecting total circulating 25OH-D, it was widely used by investigators to conduct all of the research related to circulating 25OH-D levels in various disorders. In 2004, DiaSorin introduced a chemiluminescence assay to be used on Liaison analyzer. The antibody used in this assay was similar to the one used in RIA but the sample extraction step was missing. In 2007, The Liaison Total was introduced with the improved sensitivity and specificity (23). The Liaison Total is a non-extraction assay using the proprietary technique to displace 25OH-D from the binding protein. Both assays claimed co-specificity for 25-hydroxyvitamin D3 and 25-hydroxyvitamin D2 in DEQAS survey but the 3-epimer of 25OH-D was not detectable by either assays (23).

RIA from Immunodiagnostic Systems (IDS) uses an antibody, which has only 75% of cross-reactivity with 25-hydroxyvitamin D2. Acetonitrile is used for sample extraction. IDS has also produced non-extraction enzyme immunoassay (EIA) using the same antibody. In 2009, IDS introduced a chemiluminescence method for use on their new iSYS automated analyzer. In this assay, standards based on equine serum were applied. According to manufacturers, the assay has co-specificity for 25-hydroxyvitamin D3 and 25-hydroxyvitamin D2. It was also approved by FDA for use in the USA. It is a non-extraction assay and the involvement of pH shift exists for displacement of 25OH-D from binding proteins. It was also established that IDS assays could not detect 3-epimer of 25OH-D (23).

Other than Diasorin chemiluminescence assay, Nichols Institute Diagnostics, Roche Diagnostics, Abbott and Siemens introduced 25OH-D reagents for use in random access automated instruments (27, 28). In 2001, Nichols Diagnostics introduced fully automated chemiluminescence Advantage 25OH-D assay. In this assay system, unextracted serum or plasma sample is directly added into the mixture containing human vitamin D binding protein (DBP), acridinium-ester labeled anti-DBP and 25OH-D3 coated magnetic particles (25). This assay was CPB assay and resembled the procedure described by Belsey et al (29). The differences between these methods were deproteinization of samples with ethanol and the preparation of calibrators in ethanol in the Belsey assay, whereas calibrators were prepared in serum based matrix in the Advantage assay (25, 29). It was shown that the Advantage assay constantly over estimated total 25OH-D levels and was unable to detect 25OH-D2 levels (26). In 2006, the assay was withdrawn from the market (25).

The first commercial direct automated immunoassay for 25OH-D3 was introduced by Roche Diagnostics on Elecsys and Cobas systems in 2007 (25, 30). The assay is a direct electrochemiluminescence immunoassay for human serum or plasma. It is a competitive assay in which the binding protein of vitamin D is inactivated during incubation. The assay employs polyclonal antibody directed against 25-OH vitamin D3 (39).

The Roche Vitamin D Total assay for Elecsys analyzers and Cobas Modular platforms was launched on May 13, 2011 (www.roche.com). The Elecsys Vitamin D Total assay is a fully automated assay based on biotin-streptavidin technology, and it measures both 25OH-D2 and 25OH-D3. In October 19, 2011, FDA approved the Siemens Healthcare Diagnostics Vitamin D Total assay for use on ADVIA Centaur®/XP Immunoassay Systems (www.medical.siemens.com). Abbott announced, on November 30, 2011, that it was granted approval from FDA for fully automated 25OH-D assay performed on its widely used ARCHITECT® platform. The ARCHITECT 25OH-D assay is a chemiluminescent microparticle immunoassay (CMIA) for quantitative determination of 25OH-D in human serum or plasma. (www.abbott.com).

Direct Detection Methods

HPLC procedures were developed for determination of the circulating 25OH-D, (31–33). The HPLC methods were able to separate and quantitate 25OH-D2 and 25OH-D3 levels. HPLC followed by UV detection was highly repeatable and most of the researchers consider HPLC methods golden standard (25).

The analyses of the circulating 25OH-D and its metabolites were also attempted by means of liquid chromatography/tandem mass spectrometry (LC-MSMS) (34–40). As LC-MSMS has been increasingly used in clinical laboratories, many different methodologies are being used and it has been observed that

the measurements are not straightforward. The discrepancies of the results could be attributed to variables in sample preparations, chromatography and ionization and fragmentation (41, 42).

Which method to use for Vitamin D analysis?

The most reliable assessment of vitamin D status is a measurement of plasma 25OH-D concentration. As two distinct forms of 25OH-D exist, 25OH-D3 is the major metabolite of interest, which maintains 25OH-D concentrations to a higher degree in comparison to 25OH-D2 which is solely derived from supplementation or fortification of food (15). Nearly 85% of all 25OH-D is bound to vitamin D binding protein, 15% bound to albumin and only 0.03% is free. Chromatographic separation techniques thus require an extraction step to release 25OH-D from the binding protein. Because of the lipophilic nature of 25OH-D, non-extraction methods are susceptible to matrix effects. These factors make the routine measurement of 25OH-D an analytical challenge (43).

The number of assessments of the circulating 25OH-D for diagnostic purposes has increased significantly in recent years. 25OH-D testing volumes continue to grow, making it one of the most requested assays in clinical laboratories. Dramatic increase in requests prompts many laboratories to consider using more automated assays. The replacement of traditional RIA with non-isotopically labeled assays allowed automation of the analysis, but recent studies have suggested that both the Nichols Advantage automated chemiluminescence protein-binding assay and, to a lesser extent, the IDS RIA underrecover 25-OH D2 compared with HPLC analysis (23, 37, 44). Recent publications have highlighted the interlaboratory variability of 25-OH D analysis on patient samples measured by RIA and chemiluminescence assays (45). Mass spectrometry has been the privileged research methodology and rarely been applied to the routine quantification of analytes in the routine clinical settings, but recently it has been shown that LC-MSMS offers an alternative method to traditional assays and higher specificity and sensitivity than many assays. Many LC-MSMS methods have been described for circulating metabolites of vitamin D and suggested as a candidate reference method for circulating 25OH-D3 (36).

Many authors compare commercially available 25OH-D methods with LC-MSMS accepted as a reference method. Roth et al. compared six routinely available methods; HPLC, IDS-RIA, IDS-EIA, Advantage, two versions of DiaSorin automated immunoassay; Liaison 1, Liaison 2 and Elecsys assay with LC-MSMS (15). It was observed that all evaluated methods, except HPLC, revealed considerable deviations of the individual values compared with LC-MSMS defined target values (15). Snellman et al. (46) investigated the performances of three common commercially available assays. HPLC-atmospheric pressure chemical ionization–mass spectrometry (HPLC-APCI-MS), RIA and chemiluminescent immunoassay (CLIA) methods were used. The strongest correlation was found for HPLC-APCI-MS and lowest for CLIA (46). Farrell et al. (47) compared the performance of recently launched automated immunoassays, pre-existing assays with two different LC-MSMS methods. Randomly selected patient samples were measured by two LC-MSMS methods, a RIA (DiasSorin), automated immunoassays from Abbott (Architect), DiaSorin (Liaison), IDS (ISYS), Roche (E170, monoclonal 25OH-D3 assay) and Siemens (Centaur). Although most assays have demonstrated good intra- and inter-assay precision, the automated immunoassays have demonstrated variable performance and failed to meet pre-defined performance goals. Only RIA assay showed a performance comparable to LC-MSMS (47). Van den Ouweland et al. (47) compared LC-MSMS assay with DiaSorin RIA and re-standardized version of the electrochemiluminescent immunoassay (ECLIA) from Roche Diagnostics. It was found that the DiaSorin RIA correlated well with LC-MSMS method, whereas Roche ECLIA method disagreed (48).

Because of the increased demand for vitamin D testing, laboratories shift to automated 25OH-D assays but this shift leads to significant impact on results, diagnostic classification and treatment options. Barake et al. (49) described their experience in analyzing the 25OH-D levels by using IDS-RIA and DiaSorin Liaison assays. The results revealed that 25OH-D levels were lower when the samples were analyzed by Liaison than by IDS-RIA (49). Such inter-assay variability leads to misdiagnosis of patients and target treatment thresholds need to be established (50).

Choosing an assay platform is important both for clinical laboratory professionals and researchers, and several factors affect this process. The higher throughput clinical laboratories could choose manual RIA platforms, whereas automated immunoassay platforms or automated LC-MSMS platforms are required and suitable for the highest throughput reference laboratories (25). An important factor to be considered is the recognition of the commercial assays capable of analyzing both vitamin D2 and D3. Binkley et al. (45) evaluated interlaboratory variability in serum 25OH-D results. Some assays have been found to be unable to measure reliable 25OH-D2 levels essential for the monitorization of ergocalciferol treatment (27). The International Vitamin D Quality Assessment Scheme (DEQAS) has been monitoring the performance of 25OH-D assays since 1989 (27). DEQAS demonstrated that in the samples containing only 25OH-D3, most commercial methods produced results closer to target values and the results were

highly operator-dependent (27). In the samples containing more 25OH-D2, Nichols and IDS RIA produced significantly lower results than those by other methods (27).

Because of the discrepancies between the results of assays used to measure 25OH-D levels, an international standardization of vitamin D measurements was required. For this reason, the National Institute of Standards and Technology (NIST) developed a standard reference material (SRM) for circulating vitamin D analysis. NIST measures vitamin D by isotope-dilution liquid chromatography-mass spectrometry and tandem mass spectrometry (50). SRM 972, vitamin D in human serum consists of four blood sample pools with varying levels of 25OH-D. It has certified values for 25OH-D2, 25OH-D3 and 3-epi-25OH-D3 (www.nist.gov). SRM can be used to validate new analytic methods and to designate values to in-house quality control materials. Moreover, SRM can also serve as adjunct to existing DEQAS for vitamin D analysis (51).

Conclusion

There are differences in the accuracy of methods in the steps of sample purification before final quantification or immunologic reactions. Standardization of methods for the quantification of 25OH-D by using the human-based samples would reduce the inter-method variability. The best way for laboratories to demonstrate the accuracy of their results is by participating in an external quality assessment scheme. Standardization of the assays is also required to provide clinicians with the accurate tools to diagnose hypovitaminosis. In addition, assay -specific decision limits are needed to define appropriate thresholds of treatment.

Conflict of interest statement

The authors stated that there are no conflicts of interest regarding the publication of this article.

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