Review

Current Methods for Routine Clinical Laboratory Testing of Vitamin D Levels

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ABSTRACT

Requests for vitamin D testing have increased considerably over the past few years, due in part to recent studies that show its association with protecting against skeletal and nonskeletal disorders such as malignancies and metabolic diseases. The Institute of Medicine recently created a consensus report of recommended reference rages and recommended dietary allowances for this vitamin. Two basic categories of laboratory methods exist for analysis of vitamin D; however, their differing methodologies measure a variety of forms and metabolites of vitamin D that may create confusion in interpretation of results. This review of methods considers that total 25-hydroxyvitamin D is the recommended test for vitamin D status and compares methods from published studies and recent findings of proficiency testing surveys.

Keywords: vitamin D, 25-hydroxy vitamin D, chemiluminescent immunoassay, high-performance liquid chromatography, radioimmunoassay

Previously considered an esoteric test, vitamin D has recently become one of the most talked-about analytes in clinical chemistry. According to the Mayo Clinic,[1] the recommended dosage for vitamin D to lower risk for cardiovascular disease or colon cancer is 1000 IU taken by mouth daily, particularly during periods of low sunlight exposure. This dosage can be supplied by over-the-counter supplements; larger doses are required to treat osteoporosis. The 2010 consensus report from the Institute of Medicine (IOM)[2] set the dietary reference intake for people younger than age 70 years as 600 IU/day to maintain sufficient levels of total 25-hydroxy vitamin D, with 4000 IU/day as the upper limit for intake.

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Abbreviations

IOM, Institute of Medicine; PTH, parathyroid hormone; LC-MS/MS, liquid chromatography–mass spectrometry; HPLC, high-performance liquid chromatography; RIA, radioimmunoassay; APPI, atmospheric pressure photo ionization; FDA, United States Food and Drug Administration; CLIA, chemiluminescent immunoassay; CMIA, chemiluminescentmicroparticle immunoassay; ECL, electrochemiluminescence; CV, coefficient of variation; CAP, College of American Pathologists

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Clinical Significance

Historically, vitamin D has been known for its role in the mineralization of teeth and bones through regulation of calcium and phosphorus homeostasis. More recently, there is emerging evidence of the role of vitamin D in protection against risk for malignant neoplasms,[3] cardiovascular disease, and diabetes, along with osteoporosis and other bone disorders.[4] Thus, progression of skeletal and nonskeletal diseases may be influenced by circulating levels of vitamin D, based on the discovery of more than 2000 genes in the human genome that respond to vitamin D.[5] Redefining what is considered to be a sufficient plasma level of 25-hydroxy vitamin D would potentially reclassify more people as vitamin D insufficient and trigger the need for treatment and monitoring of vitamin D levels.[6] The relationship of blood vitamin D levels to these disorders is not entirely clear, so standardization of laboratory methods for vitamin D analysis and redefining the reference ranges that indicate health and disease are of utmost importance.

During the past decade, deficiency of total 25-hydroxy vitamin D has been defined as less than 10 ng/mL and classification of sufficient levels as being between 30 and 100 ng/mL. For 1,25-dihydroxy vitamin D, sufficient levels are between 16 and 56 ng/mL, with levels of greater than 100 ng/mL considered to be toxic.[7] Based on data gathered from an informal survey of reference laboratories participating in 2009 CAP proficiency testing

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surveys,[8] a variety of ranges for total vitamin D were being used as medical decision limits. Also, many physicians consider that parathyroid hormone (PTH) levels should be assessed along with levels of vitamin D when making therapeutic decisions.[8] In 2010, new guidelines from the IOM[9] redefined sufficient levels of total vitamin D as 20 to 50 ng/mL and toxicity as greater than 50 ng/mL. Evidence shows that total 25-hydroxy vitamin D is the most clinically significant form.[10,11] The methods used in routine laboratory testing should be able to measure total 25-hydroxy vitamin D and to convey that information clearly to clinicians.

Background

Vitamin D is a steroidal hormone that requires multiple metabolic steps to become active and useful in the human body. Vitamin D can be found in several sources, including fish and eggs, and exists as vitamin D_2, or 25-hydroxy vitamin D_2 (ergocalciferol) and 25-hydroxy vitamin D_3 (cholecalciferol). The latter is produced in the skin from 7-dehydrocholesterol after UV irradiation. Both forms are present in low concentrations in foods and biological tissues and are also available in supplements and multivitamins.

25-hydroxy vitamin D, which is produced endogenously and exogenously, is converted to the biologically active form, 1,25-dihydroxy vitamin D (calcitriol), in the kidneys. In turn, this substance is catabolized to a variety of compounds that may appear in measurable amounts in tissue. The tissue distribution of vitamin D metabolites has not been well characterized due to problems in methodologies.[12] Metabolism of vitamin D to inactive forms takes place primarily through oxidative reactions and results in a variety of compounds. Regardless, only 25-hydroxy vitamin D and 1,25-dihydroxyvitamin D have clinical significance based on the findings of the IOM and other organizations and researchers, with total 25-hydroxy vitamin D being the most significant for therapeutic decisions.[2,10,11] An additional vitamin D type, 25-hydroxy vitamin D_3 epimer (C-3 epimer), was originally found in children less than age 1 year[13] but has since been found in adult plasma samples as well.[14] This form of vitamin D is measured differently by various methods but typically is distinguished separately by liquid chromatography–mass spectrometry (LC-MS/MS).[15] It is also clear that vitamin D is referred to by a variety of names due to different testing methods.

Methods of Vitamin D Measurement

Historically, vitamin D was measured by competitive binding methods, high-performance liquid chromatography (HPLC), and radioimmunoassay (RIA). A commonly used RIA kit, developed by DiaSorin S.p.A (Saluggia, Italy) was the method used by many reference laboratories and is considered the gold standard.[7,16] This method has been used to establish reference ranges during the past decade.[7] The DiaSorin 25-hydroxy vitamin D assay is a 2-step procedure that involves a rapid extraction of 25-hydroxy vitamin D and other hydroxylated metabolites from serum or plasma, followed by a competitive RIA procedure using an antibody with specificity for 25-hydroxy vitamin D.

The reference method for vitamin D analysis has been LC-MS/ MS, which can measure vitamin D_2, vitamin D_3, and the D_3 epimer separately; through calculation, total vitamin D is reported.[7] This method was chosen by the Nutritional Laboratory at the Centers for Disease Control and Prevention (CDC) and the National Laboratory in the United Kingdom for analysis of vitamin D for health and nutrition surveys, partly due to its ability to distinguish the various forms of vitamin D in plasma that may be found in people of all ages.[17,18,19] These methods tend to be labor intensive and technically difficult.

High-performance liquid chromatography methods quantitate 25-hydroxy vitamin D_2 and D_3. HPLC methods are available in kit form (Hitachi High-Technologies Corporation Tokyo, Japan, and Thermo Fisher Scientific, Sunnyvale, CA) in an effort to standardize test quality and to make the assays more cost effective and less labor intensive. The Hitachi method uses a reverse phase column and diode array detection, which allow for highly sensitive simultaneous analysis at optimal wavelengths. This method can be used to analyze food and biological samples.

Newer chromatography methods have been developed to improve sensitivity, to simplify steps, and to measure all forms of vitamin D. One example is an LC-MS/MS method that was developed to analyze all forms and metabolites of vitamin D simultaneously, including D_2, D_3, and 25-hydroxy vitamin D in serum. The process uses an ionization detector technique known as atmospheric pressure photo ionization (APPI) to provide additional

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sensitivity for analysis. The method is less difficult compared with other LC methods because it doesn't require preconcentration steps.[12]

Although most LC-MS methods can separate and quantify vitamin D_2 and vitamin D_3, most immunoassays do not. Depending on the specificity of the antibody used in the immunoassay method, some immunoassays measure only one form, some measure both forms equally (ie, DiSorin RIA), and others measure vitamin D_2 and D_3 with different cross-reactivity of the assay.[15,20]

Immunoassay Methods

Several United States Food and Drug Administration (FDA)–approved immunoassay methods are available, including quantitative chemiluminescent immunoassay (CLIA) methods. DiaSorin, the manufacturer of the commonly used RIA method, also offers a CLIA method for its LIAISON platform. The method, developed in 2002, measures total 25-hydroxy vitamin D and other hydroxylated vitamin D metabolites in human serum. During the first step, 25-hydroxy vitamin D is dissociated from its binding protein and binds to the specific solid phase antibody, followed by the addition of vitamin D-isoluminol tracer; unbound material is removed with a wash cycle. In the next step, the reagents are added to initiate the chemiluminescent reaction. The light signal is detected by a photomultiplier as relative light units; this measurement is inversely proportional to the concentration of 25-hydroxy vitamin D.[21] Proficiency testing surveys conducted in 2009 reported that more than a third of the laboratories that responded were using the DiaSorin LIAISON method for measuring total vitamin D levels.[22]

Abbott Laboratories (Abbott Park, IL) offers a fully automated immunoassay for 25-hydroxy vitamin D on the ARCHITECT platform. The assay is a 1-step delayed chemiluminescentmicroparticle immunoassay (CMIA) with an automated online pretreatment step designed to allow vitamin D assays into routine laboratory testing workflow.[23] This method received FDA approval in 2011.[24]

ImmunoDiagnostics Inc. (Woburn, MA) offers an automated CMIA immunoassay method, the IDS–iSYS, for the quantitative determination of total 25-hydroxyvitamin D and other hydroxylated metabolites in human serum or plasma. It reports equal specificity for 25-hydroxy vitamin D_3 and D_2 and sensitivity to 5.5 ng per mL.[25]

The patented electrochemiluminescence (ECL) method by F. Hoffman-La Roche AG (Basel, Switzerland) for the cobas platform offers a 25-hydroxy vitamin D assay. The test is available for use on all of the Roche cobas modular analyzer platforms; it received FDA clearance in July 2012.[26]

Enzyme immunoassay methods are also available. Diazyme Laboratories (Poway, CA) offers a method that uses a homogenous enzyme-coupled vitamin D binding protein to measure true-total levels of 25-OH vitamin D (ie, the sum of D_3 and D_2). The vitamin D binding protein recognizes vitamin D_2 and D_3 equally and also recognizes the true-total level of 25-hydroxy vitamin D.[27]

An immunoassay method is available on the ADVIA Centaur platform developed by Siemens AG (Munich, Germany). In this method, total vitamin D is assayed by a homogenous competitive immunoassay in which relative light units are detected as an endpoint.[28]

There have been many published results of comparison studies between RIA methods and HPLC,[14] and between other immunoassay methods and HPLC.[29,30,31,32,33,34] A more recent comparison[8] shows better agreement between immunoassay methods and LC-MS/MS.

Results

The results of a 2009 and 2010 CAP proficiency-testing survey[15,34] have shown that most current vitamin D assay methods provide similar absolute values, assay linearity, and assay precision. However, recent survey results from Vitamin D External Quality Assessment Scheme,[35] a United Kingdom proficiency-testing provider also show that the only assays that quantitatively detect total vitamin levels are HPLC methods, LC-MS methods, and the DiaSorin assays.[8,35]

Specificity can be an issue for immunoassay methods, especially in relation to the proportion of 25-hydroxy vitamin D that is quantified. Chromatography methods are more specific but are less convenient to perform, due to multiple processing steps. Also, HPLC and LC–MS/MS require more expensive equipment and expertise to operate but usually require lower costs for reagent

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usage. Procedures are being developed to semiautomate or automate HPLC and LC–MS/MS; however, run times remain considerably longer than for immunoassays, especially if those assays are performed on automated platforms.[8] For most HPLC and LC–MS/MS methods, losses are corrected by the inclusion of an internal standard which, in part, may account for a positive bias in results compared with immunoassays. In general, the precision levels of the immunoassay, HPLC, and LC–MS/MS are comparable; all have the required sensitivity to identify severe vitamin D deficiency.[30]

There appears to be a positive bias in results using the LC-MS/MS methods and a slight negative bias with the DiaSorin LIASON method. This can result in discrepancies in interpreting results compared with the medical-decision limit, in that patients are more likely to be classified as vitamin D inadequate or deficient by the DiaSorin LIAISON method compared with an LC-MS/MS method.[31]

A recent study[32] was conducted to test the performance of 5 automated immunoassays (the Abbott ARCHITECT, DiaSorin LIAISON, IDS i-SYS, Roche [E170, monoclonal 25-hydroxy vitamin D_3 assay], and Siemens ADVIA Centaur), an RIA (DiaSorin), and 2 LC-MS/MS methods. The automated immunoassay methods showed variability; the LC-MS/MS methods agreed well. The RIA yielded similar results to those from the LC-MS/MS methods, with a slight positive bias. All immunoassays measured total 25-hydroxy vitamin D levels, with the exception of the Roche assay, which measured only vitamin D_3 levels. The mean positive bias was the highest with the Abbott Laboratories method. The Roche 25-hydroxy vitamin D_3 assay demonstrated a small mean bias. Most assays demonstrated good intra- and interassay precision, with a coefficient of variation (CV) of less than 10%. Recent College of American Pathologists (CAP) proficiency testing results showed inaccuracy with a negative bias (ie, <75% of the target value) on multiple samples for the Siemens ADVIA Centaur method, whereas the results obtained using the Abbott ARCHITECT and the Diasorin LIAISON methods were within 25% of the target value.[15]

Holmes and his research team[33] compared results from the Abbot ARCHITECT and the Siemens ADVIA Centaur immunoassay methods for 25-hydroxy vitamin D levels against results obtained by LC-MS/MS. Both of the immunoassays showed positive bias, which results in overestimating vitamin D deficiency; this could lead to overtreatment.

Discussion

Earlier method comparisons of HPLC and immunoassay methods showed significant discrepancies in results, causing inaccuracy in the immunoassay methods when compared with the reference method.[19,20] However, improvements in methodologies result in better correlation among results when comparing methods used in proficiency testing.[8] A 2005 study[29] showed good agreement between the results with the DiaSorin LIASON method and those obtained with an LC-MS/MS method.

Conclusion

Automated immunoassays are available for total vitamin D levels and have improved in precision and accuracy due to market demands. They are integrated with existing platforms and provide rapid turnaround time; unlike chromatography methods, they do not required specialized expertise for testing. The DiaSorin LIASON and Abbott ARCHITECT methods have performed reasonably well with accuracy and precision tests, based on the results of a 2009 proficiency-testing survey.[34] These methods performed with poorer accuracy in the 2012 proficiency-testing survey, whereas the IDS i-SYS performed with greater accuracy.[15]

An increasing number of patients are being advised to take vitamin D supplements or even to receive higher dosages of vitamin D through therapeutic treatment to lower their risk for skeletal and nonskeletal diseases, such as malignant neoplasms or cardiovascular disease. However, the IOM[2] reported in 2010 the recommended daily allowance to be 600 IU/day for most patients and has established that toxicity may be reached at lower circulating levels of vitamin D than previously thought. Therefore, precise and accurate test methods are crucial to distinguish insufficient levels based on undertreatment and toxic levels based on overtreatment. Given the variety of names for vitamin D and its multiple forms and products, it may become confusing for health care professionals to know which to order. The confusion is further compounded by the fact that many referring laboratories list different names when ordering vitamin D laboratory tests. Therefore, it is important that laboratory professionals are able to assist clinicians in ordering and interpreting vitamin D tests. LM

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