Blood biomarkers of vitamin D status\textsuperscript{1–4}

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ABSTRACT
In the past quarter century, more than 50 metabolites of vitamin D have been described. To date, only a few of these have been quantified in blood, but this has widened our understanding of the pathologic role that altered vitamin D metabolism plays in the development of diseases of calcium homeostasis. Currently, awareness is growing of the prevalence of vitamin D insufficiency in the general population in association with an increased risk of several diseases. However, for many researchers, it is not clear which vitamin D metabolites should be quantified and what the information gained from such an analysis tells us. Only 2 metabolites, namely, 25-hydroxyvitamin D [25(OH)D] and 1,25-dihydroxyvitamin D [1,25(OH)\textsubscript{2}D], have received the greatest attention. Of these, the need for measuring serum 1,25(OH)\textsubscript{2}D is limited, and this metabolite should therefore not be considered as part of the standard vitamin D testing regimen. On the other hand, serum 25(OH)D provides the single best assessment of vitamin D status and thus should be the only vitamin D assay typically performed. Currently, numerous formats exist for measuring serum 25(OH)D concentrations, each with its own advantages and disadvantages. This article reviews the currently available methods for serum 25(OH)D quantitation and considers important issues such as whether both the D\textsubscript{2} and the D\textsubscript{3} forms of the vitamin should be assayed, whether total or free concentrations are most important, and what measures should be taken to ensure the fidelity of the measurements. *Am J Clin Nutr* 2008;87(suppl):1087S–91S.

INTRODUCTION
It has been >80 y since the discovery of vitamin D and its ability to cure rickets in children. The central role this secosteroid plays in calcium and phosphate homeostasis is well appreciated. However, recent epidemiologic and clinical studies have indicated that vitamin D may have far greater actions than to solely prevent rickets in children. Diseases such as osteoporosis, muscle weakness, several types of cancer, diabetes, hypertension, and cardiovascular disease may result from subtle and chronic vitamin D deficiency (1). Indeed, recent data from the third National Health and Nutrition Examination Survey concluded that nearly 90% of women aged >70 y did not meet the recommended daily intake of vitamin D and were at risk of developing vitamin D deficiency (2). With a new appreciation of such widespread vitamin D deficiency and its potential impact on health, awareness is growing of the need for accurate assessment of vitamin D status in the general population as well as in large populations typically found in epidemiologic studies. Although >50 different vitamin D metabolites have been reported to date, vitamin D assay methods have focused primarily on quantitating the parent sterol vitamin D and its metabolites 25-hydroxyvitamin D [25(OH)D] and 1,25-dihydroxyvitamin D [1,25(OH)\textsubscript{2}D]. This article will address which vitamin D metabolites should be measured and what analytic methods are available for performing this analysis.

WHICH VITAMIN D METABOLITES SHOULD BE MEASURED?
The development of sensitive and specific assays for vitamin D, 25(OH)D, and 1,25(OH)\textsubscript{2}D has proved to be an invaluable asset in the research laboratory in defining vitamin D action and several diseases that result from altered vitamin D metabolism. The development and application of these assays to large population-based studies or in the clinical biochemistry laboratory has proceeded much more slowly. This has been due, in large part, to the inherent problems associated with these assays and the detection of these vitamin D metabolites that circulate in the nanomolar to micromolar concentration range.

Another complicating factor is the presence of 2 forms of vitamin D. Vitamin D\textsubscript{2}, or cholecalciferol, is produced from the action of ultraviolet radiation on the skin and represents the most important source of vitamin D in humans. Only a few foods naturally contain vitamin D\textsubscript{3}. Intake from the diet or in the form of supplements can be either as vitamin D\textsubscript{3} or as a closely related molecule, vitamin D\textsubscript{2} (ergocalciferol). This form of vitamin D is produced from the irradiation of ergosterol. Although some of the currently available assays for vitamin D metabolites can provide a measure of both the D\textsubscript{2} and D\textsubscript{3} forms in the circulation, separate assessment of both forms of vitamin D metabolites in large epidemiologic studies is usually not performed, and only the total vitamin D metabolite concentration is measured.

There are exceptions to this, however. For example, in studies designed to assess the efficacy of vitamin D supplementation with the D\textsubscript{2} form or to examine the contribution of both the D\textsubscript{2} and D\textsubscript{3} forms in the circulation, separate assessment of both forms of vitamin D metabolites should be performed.
the D₃ forms to total vitamin D status, it would be necessary to measure both the D₂ and the D₃ metabolites of the vitamin in the circulation. This is especially important when assessing the serum 25(OH)D response to vitamin D₂ administration, because several studies have suggested that the D₂ form of the vitamin is much less effective than is vitamin D₃, in raising serum 25(OH)D over an extended dosing interval (3–5). This is believed to be due to a lesser affinity of 25(OH)D₂ to the vitamin D binding protein (DBP) than that of 25(OH)D₃, resulting in a more rapid clearance of 25(OH)D₂ (6). However, for most epidemiologic studies concerned with only an assessment of overall vitamin D status, it would be necessary only to measure total vitamin D. Thus, most of this discussion will focus on total vitamin D quantitation without consideration of separately assaying the 2 forms of vitamin D.

Another issue to be addressed is whether there is clinical utility in measuring the free vitamin D metabolite concentration. Most circulating 25(OH)D and 1,25(OH)₂D₃ is transported in the circulation bound to DBP (80–90%) and to albumin (10–20%). A very small fraction [0.02–0.05% of 25(OH)D and 0.2–0.6% of total 1,25(OH)₂D₃] remains free or unbound (7). Thus, under physiologic conditions, nearly all circulating vitamin D compounds are protein bound. However, the vitamin D–DBP complex can be taken up by target cells via an endocytic process involving megalin and cubulin (8). Once in the cell, the DBP is proteolytically degraded, leaving the intracellular vitamin D metabolite available for further action or metabolism. Furthermore, free vitamin D metabolite concentrations appear to be well maintained, even in subjects with liver disease and reduced DBP concentrations, which suggests that there is little utility in assessing free vitamin D metabolite concentrations as indicators of vitamin D status.

Free serum 25(OH)D and 1,25(OH)₂D can be directly measured by centrifugal ultrafiltration or equilibrium dialysis (7). Free plasma 25(OH)D and 1,25(OH)₂D concentrations can also be determined by calculation provided that both the total vitamin D metabolite and DBP concentrations are known as well as the affinity constants of albumin and DBP for the respective vitamin D metabolites (9). Because of the lack of data showing that free 25(OH)D or 1,25(OH)₂D concentrations are a better predictor of vitamin D deficiency, assessment of total vitamin D metabolite concentration is sufficient in most clinical settings.

Another concern in selecting which vitamin D metabolite should be measured is whether there is clinical utility in measuring the free vitamin D metabolite concentration. Most circulating 25(OH)D and 1,25(OH)₂D₃ is transported in the circulation bound to DBP (80–90%) and to albumin (10–20%). A very small fraction [0.02–0.05% of 25(OH)D and 0.2–0.6% of total 1,25(OH)₂D₃] remains free or unbound (7). Thus, under physiologic conditions, nearly all circulating vitamin D compounds are protein bound. However, the vitamin D–DBP complex can be taken up by target cells via an endocytic process involving megalin and cubulin (8). Once in the cell, the DBP is proteolytically degraded, leaving the intracellular vitamin D metabolite available for further action or metabolism. Furthermore, free vitamin D metabolite concentrations appear to be well maintained, even in subjects with liver disease and reduced DBP concentrations, which suggests that there is little utility in assessing free vitamin D metabolite concentrations as indicators of vitamin D status.

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Values obtained from HPLC analysis, this assay has been approved by the Food and Drug Administration for clinical use in the United States (DiaSorin, Stillwater, MN). The antibody used in this equilibrium RIA recognizes both 25(OH)D$_2$ and 25(OH)D$_3$ as well as other hydroxylated metabolites, such as 24,25-dihydroxyvitamin D; 25,26-dihydroxyvitamin D; and 25(OH)D$_{26,23}$-lactone. However, the overall contribution of these latter metabolites to the circulation is rather small (≤6%) and thus their contribution can be ignored. A second 25(OH)D RIA is also available from IDS Inc (Immunodiagnostic Systems Inc, Fountain Hills, AZ). It is similar to the DiaSorin assay but uses a 2-step extraction procedure. Portions of the extracted samples, calibrators, and controls are incubated with $^{125}$I-25(OH)D tracer and a highly specific sheep anti-25(OH)D polyclonal antibody. Separation of antibody-bound tracer from free is achieved by a short incubation with Sac-Cel. The antibody is reported to have 75% cross-reactivity with 25(OH)D$_2$. This assay has been shown to correlate well with values obtained by HPLC ($r^2 = 0.89$) and by the DiaSorin RIA ($r^2 = 0.92$).

There are also 2 enzyme-linked immunosorbent assay (ELISA) format assays for 25(OH)D that are commercially available. The IDS Inc assay used biotin-labeled 25(OH)D, which is added to the calibrators, controls, and 25 μL of nonextracted serum. The diluted samples are then incubated in microtiter wells that are coated with a sheep antibody to 25(OH)D. After the wells are washed, color development is accomplished via incubation with horseradish peroxidase–labeled avidin and tetramethylbenzidine as the chromogenic substrate. This antibody (probably the same as in their RIA) has 75% cross-reactivity with 25(OH)D$_3$. The second ELISA assay (Immunodiagnostik) is similar to the Immunodiagnostik competitive protein binding assay that uses the DBP described above. However, in this direct assay, standards, controls, and patient samples are incubated with a releasing agent to free the 25(OH)D from the DBP. An aliquot from each of these extractions is transferred to a microplate that has been coated with 25(OH)D, and an antibody to 25(OH)D is added. After an overnight incubation step, 25(OH)D in the sample and a fixed amount of 25(OH)D bound to the microtiter well compete for the binding of the antibody. A peroxidase-conjugated secondary antibody is added, and the complex is detected after the addition of tetramethylbenzidine. Interestingly, the antibody used in this assay has only 23% cross-reactivity with 25(OH)D$_2$.

Two other 25(OH)D assay formats deserve mention. DiaSorin has developed a chemiluminescent 25(OH)D assay that is fully automated on the LIAISON analyzer (Stratec Biomedical Systems, Birkenfeld, Germany). Under this format, a 25(OH)D-chemiluminescent tracer competes with 25(OH)D in the sample for binding to antisera-coated on paramagnetic beads. The polyclonal antisera is the same as that used in the DiaSorin 25(OH)D RIA (described above). This antisera has equal affinity for both the D$_2$ and the D$_3$ forms of the vitamin D metabolites, and the assay provides total 25(OH)D values. The method does not require any pretreatment of samples. Assay time is 40 min, and ≈90 samples per hour can be analyzed. When compared against the DiaSorin RIA, a correlation coefficient of 0.94 was obtained; when compared against liquid chromatography–tandem mass spectrometry (LC-MS/MS), the correlation coefficient was 0.95 (18).

The last method, LC-MS/MS, is considered to be the most accurate of all methods to date and is currently regarded as the gold standard. LC-MS/MS can separate and accurately quantify both 25(OH)D$_2$ and 25(OH)D$_3$. In addition, this method has been shown to correlate well with the DiaSorin RIA ($r = 0.74$ to 0.96) (19, 20). However, all of the LC-MS/MS techniques require derivatization or deuterated internal standards. In addition, LC-MS/MS methods frequently encounter issues with ion suppression. Ion suppression represents difficulties with reproducibility and accuracy when analyzing small quantities of analytes in complex samples such as biological fluids. It may result from co-eluted matrix components, affecting the detection capability, precision, or accuracy of the analytes of interest. This change typically is observed as a loss in response, thus the term ionization suppression. It frequently occurs when internal standards are used that do not share chemical and structural properties with vitamin D. Thus, it is critical to minimize or compensate for ion suppression if found to be present. A recently described method used a novel internal standard, deuterated $^\Lambda$-tetrahydrocannabinol-D$_3$, to minimize ion suppression with shorter analysis times (19). Deuterated vitamin D compounds are also now available as suitable internal standards (20). Generally speaking, this assay method is best left to high-volume reference laboratories. A summary of the serum 25(OH)D assay platforms is provided in Table 1.

WHICH ASSAY PLATFORM SHOULD BE USED?

Deciding which vitamin D assay format to use can be a daunting task. As discussed above, assays for 25(OH)D are available in several platforms. Although establishing an in-house assay might be the most cost-effective means of performing 25(OH)D assays, without the appropriate equipment and expertise, such an undertaking can be formidable. Rather, one of the commercially available RIA or ELISA procedures may be suitable for use in the investigator’s laboratory. Alternatively, if the research budget allows, investigators could obtain a quote from one of the many commercial laboratories performing this assay. Under this setting, the price per test typically diminishes as the number of samples increases.

Quality control concerns for each of the assays must also be considered, particularly in light of a recent report that highlighted interlaboratory variability in serum 25(OH)D results (21). Those authors reported unacceptable variation in circulating 25(OH)D measurements encountered from laboratory-to-laboratory as well as method-to-method and the confounding of the 2. For example, 42 specimens from postmenopausal women were sent to a commercial laboratory that used acetonitrile extraction and their own in-house RIA. Of the 42 patients, 17% were classified as being vitamin D insufficient [serum 25(OH)D < 80 nmol/L]. Twenty different specimens from a nearly identical group of women went to laboratory B, which used a commercially available 25(OH)D RIA. In this case, 90% of the women were classified as being vitamin D insufficient on the basis of the serum 25(OH)D value. It is not surprising that discordant results were obtained, because these laboratories used substantially different methods to measure serum 25(OH)D. Certainly, the variability among the methods to detect 25(OH)D$_2$ may explain some of the differences. However, another likely source of variability may have been inaccurate preparation of the standards used in the assays. Although most (but not all) of the commercially available assay kits (RIA and ELISA) will contain the manufacturer’s own quality control material, this will only indicate whether there has
been a general problem with the assay performance and does not guarantee that the result is accurate simply because the control value came within the specified range. For commercial assays not providing quality control specimens, a suitable serum control value came within the specified range. For commercial assays guarantee that the result is accurate simply because the control

<table>
<thead>
<tr>
<th>Assay type and manufacturer</th>
<th>Sample type and volume</th>
<th>Extraction</th>
<th>Range of detection</th>
<th>Sensitivity</th>
<th>Intraassay CV</th>
<th>Interassay CV</th>
<th>Assay time</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>RIA DiaSorin</td>
<td>Serum or plasma, 50 μL</td>
<td>Acetonitrile</td>
<td>0–100</td>
<td>≤6</td>
<td>&lt;8</td>
<td>&lt;12</td>
<td>2.2 h</td>
<td>Calibrators and controls in serum matrix; no yield determination required</td>
</tr>
<tr>
<td>ELISA IDS Inc</td>
<td>Serum or plasma, 50 μL</td>
<td>None</td>
<td>4–400</td>
<td>≤3</td>
<td>6.8</td>
<td>8.9</td>
<td>3 h</td>
<td>Calibrators and controls in serum matrix; no yield determination required; 75% cross-reactivity for 25(OH)D2</td>
</tr>
<tr>
<td>Immunodiagnostic</td>
<td>Serum or plasma, 25 μL</td>
<td>Proprietary extraction reagent</td>
<td>6.3–250</td>
<td>2</td>
<td>10</td>
<td>8</td>
<td>ON</td>
<td>Monoclonal antibody; 23% cross-reactivity for 25(OH)D2</td>
</tr>
<tr>
<td>CPB Immunodiagnostic</td>
<td>Serum or plasma, 50 μL</td>
<td>Acetonitrile</td>
<td>6.4–250</td>
<td>5.6</td>
<td>11</td>
<td>13</td>
<td>4.5 h</td>
<td>Uses DBP in EIA format; 100% cross-reactivity for 25(OH)D2</td>
</tr>
<tr>
<td>HPLC Immunodiagnostic</td>
<td>Serum, 500 μL</td>
<td>Acetonitrile and C18 cartridge extraction</td>
<td>Up to 1250</td>
<td>4</td>
<td>5.2</td>
<td>8.4</td>
<td>20 min</td>
<td>Laboratory must have HPLC unit with silica column; 25(OH)D2 and 25(OH)D3 separated with different column</td>
</tr>
<tr>
<td>Chemiluminescence</td>
<td>Serum or plasma, 25 μL</td>
<td>Automated</td>
<td>7.5–375</td>
<td>≤10</td>
<td>4</td>
<td>6</td>
<td>40 min</td>
<td>Fully automated on Liaison instrument; 100% cross-reactivity for 25(OH)D2</td>
</tr>
</tbody>
</table>

1 RIA, radioimmunoassay; 25(OH)D3, 25-hydroxyvitamin D3; ELISA, enzyme-linked immunosorbent assay; ON, overnight; CPB, competitive protein binding; DBP, vitamin D binding protein.

SUMMARY

In the past few years, there has been an increasing awareness of the multisystem effects of vitamin D and its metabolites in humans. No longer is vitamin D regarded simply as a steroid hormone that prevents rickets in children. Rather, its roles in prevention, diabetes, motor function, and immunologic processes, to name but a few, have opened the door for large population-based studies. Such studies have aimed to specifically define the role of vitamin D in these disease states and how
alterations in vitamin D sufficiency affect the risk of development of such diseases. The success of these and other epidemiologic studies of vitamin D require the ability to accurately and reproducibly measure serum vitamin D metabolites. There has been considerable progress in vitamin D assay methods, and today researchers can measure 25(OH)D by an array of different assay techniques. The choice of which assays to use will be dictated by the availability of required equipment, technical expertise, and whether a need exists to quantitate both the D3 and D2 forms of the vitamin D metabolites. Most importantly, there exists an external quality control mechanism for ensuring the accuracy and precision of such vitamin D measurements in each researcher’s laboratory.

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REFERENCES