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Vitamin D: Marker, measurand & measurement

Niek F. Dirks[1,2], Etienne Cavalier[3], Annemieke C Heijboer[4,5,6,7*]

[1] Atalmedial Diagnostics Centre, Spaarne Gasthuis, Haarlem, The Netherlands.

[2] Department of Clinical Chemistry, Hematology and Immunology, Medical Center Alkmaar, Alkmaar, the Netherlands

[3] Department of Clinical Chemistry, University of Liège, CHU de Liège, Liège, Belgium

[4] Amsterdam UMC location Vrije Universiteit Amsterdam, Department of Clinical Chemistry, Endocrine Laboratory, Boelelaan 1117, Amsterdam, The Netherlands

[5] Amsterdam Gastroenterology, Endocrinology & Metabolism, Amsterdam, The Netherlands

[6] Amsterdam UMC location University of Amsterdam, Department of Clinical Chemistry, Endocrine Laboratory, Meibergdreef 9, Amsterdam, The Netherlands

[7] Amsterdam Reproduction & Development Research Institute, Amsterdam, The Netherlands

*Corresponding author: Annemieke C Heijboer, Amsterdam UMC, Vrije Universiteit Amsterdam, Endocrine Laboratory, Department of Clinical Chemistry, de Boelelaan 1117, Amsterdam, The Netherlands; and Amsterdam UMC, University of Amsterdam, Endocrine Laboratory, Department of Clinical Chemistry, Meibergdreef 9, Amsterdam, The Netherlands, E-mail: a.heijboer@amsterdamumc.nl

Abstract

The measurement of vitamin D metabolites aids in assessing vitamin D status and in diagnosing disorders of calcium homeostasis. Most laboratories measure total 25(OH)D, while others have taken the extra effort to measure $25(OH)D_2$ and $25(OH)D_3$ separately and additional metabolites such as 1,25-dihydroxyvitamin D and 24,25-dihydroxyvitamin D. The aim of this review is to provide an updated overview of the main markers of vitamin D metabolism, define the intended measurands and discuss the advantages and disadvantages of the two most widely used assays, automated assays and LC-MS/MS.

Whether using the easy and fast automated assays, or the more complex LC-MS/MS, one should know the pitfalls of the used technique in order to interpret the measurements. In conclusion, automated assays are unable to accurately measure 25(OH)D in all patient groups, including persons using D_2. In these cases, a LC-MS/MS method, when appropriately developed and standardized, produces a more reliable measurement.

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Introduction

The percentage of people considered vitamin D deficient is ever-growing as a consequence of a depletion of sufficient amounts of sunlight by our changing ways of life (1). Because of this, and as a consequence of the increasing variety of conditions known to be associated with vitamin D deficiency, vitamin D testing has skyrocketed. Nowadays, many laboratories, big and small, are running tests for assessment of vitamin D status. While most run the well-known 25-hydroxyvitamin D (25(OH)D) metabolite, other vitamin D metabolites may offer vital information in diagnosing the more rare conditions. When measuring any of the vitamin D metabolites, it is essential to know what the actual measurand of the assay is, as it is not always the same for every method designed to measure vitamin D. Second, knowing the pitfalls of the used assay, most often an automated immunoassay or a liquid-chromatographer coupled to tandem mass-spectrometry, is important for interpretation of the results and adequate application in specific patient groups. Here we review the different vitamin D markers that are in use today, articulate the intended measurand, and discuss the advantages and disadvantages of the two most used techniques for assessment of vitamin D status.

Metabolism

Vitamin D is not a single molecule that after production or ingestion rushes through our veins to exert its function on the target organs to maintain calcium homeostasis. In fact, it requires a whole cascade of metabolizing reactions that precede the formation of the active hormone (Figure 1) (2).

The starting compound is 7-dehydrocholesterol, a final intermediate in the cholesterol biosynthesis. The enzyme 7-dehydrocholesterol reductase uses NADH to reduce this molecule to compose cholesterol. Alternatively, when UVB radiation penetrates the epidermal layers of our skin, part of the 7-dehydrocholesterol molecule can absorb light and break open. The resulting pre-vitamin D_3 is unstable and immediately isomerizes into vitamin D_3, which then enters circulation and binds to vitamin D binding protein (VDBP). At this point,

vitamin D_2, a very similar but vegetable form of vitamin D, which we obtain from certain foods or supplements may also enter this metabolic route. Both vitamin D_3 and D_2 are only present in small amounts in circulation. Liver cytochrome P450 CYP2R1 is the main 25-hydroxylase that catalyzes the 25-hydroxylation reaction to form respectively 25-hydroxyvitamin D_3 ($25(OH)D_3$) or 25-hydroxyvitamin D_2 ($25(OH)D_2$) (3). These vitamin D metabolites are most abundant in circulation but are still not bioactive. A second hydroxylation at the C1 position step by the kidney enzyme 1α-hydroxylase yields 1,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$) or 1,25-dihydroxyvitamin D_2 ($1,25(OH)_2D_2$), both are able to bind the nuclear vitamin D receptor (VDR). The renal 1α-hydroxylation is tightly regulated by parathyroid hormone (PTH), fibroblast growth factor 23 (FGF23), calcium, phosphate and $1,25(OH)_2D$ itself. PTH upregulates the expression of 1α-hydroxylation, while FGF23 and $1,25(OH)_2D$ downregulate its expression. Apart from renal activation, many other cell types also harbor 1α-hydroxylase and are thus able to bioactivate $25(OH)D_3$ and $25(OH)D_2$ for autocrine or paracrine use (4). This amount of extra-renal bioactivation only contributes little to the circulating concentrations of total $1,25(OH)_2D$ and is not under regulation of PTH or FGF23. The 25-hydroxy and 1,25-dihydroxy forms of vitamin D may be inactivated and prepared for secretion by renal or extra-renal 24α-hydroxylase, which adds another hydroxyl group at the C24 position, resulting in respectively 24,25-dihydroxyvitamin D ($24,25(OH)_2D$) or 1,24,25-trihydroxyvitamin D ($1,24,25(OH)_3D$). This route is also under regulation of PTH and FGF23, which respectively downregulate and upregulate the expression of the gene coding for $24,25(OH)_2D$.

Another enzyme, 3-epimerase, converts the orientation of the hydroxyl group at the C3 position of small amounts of $25(OH)D$ and $1,25(OH)_2D$ resulting in 3-epi-$25(OH)D$ and 3-epi-$1,25(OH)_2D$. Only around 4% of circulating $25(OH)D$ is of the epimerized form, yet incidentally higher amounts up to 25% have been reported and may be even higher in infants up to 1 year of age (5, 6, 7, 8). The function of the epimers is uncertain, yet seems of less clinical significance as they have lower affinity for VDBP and 3-epi-$1,25(OH)_2D$ has a significantly lower affinity to the VDR (9).

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Vitamin D metabolites as biomarkers for clinical use

25(OH)D

25(OH)D is the main circulating vitamin D metabolite and forms the pool from which $1,25(OH)_2D$ can be formed by renal or extra-renal 1α-hydroxylation when required. Consequently, 25(OH)D is considered the best reflector of the body's vitamin D status as sufficient renal and local $1,25(OH)_2D$ can only be generated if sufficient 25(OH)D is available. Measurement is recommended in individuals at increased risk of vitamin D deficiency, and include obese individuals, pregnant and lactating women, older adults with a history of falls or fractures, individuals with darker skin pigmentation, patients with kidney disease, liver failure, bone disease, hyperparathyroidism, granuloma-forming disorders, lymphomas, and patients on certain medications (10). The debate on the exact target values for 25(OH)D sufficiency is still ongoing but most studies set target values somewhere between 50 and 80 nmol/L (11, 12, 13).

$1,25(OH)_2D$

Only a few rare conditions justify measuring $1,25(OH)_2D$, which have been reviewed elsewhere (10, 14). In short, disorders characterized by defective 1α-hydroxylase, such as vitamin D-dependent rickets type 1, result in an inability to produce $1,25(OH)_2D$ and thus abnormally low concentrations (below 59 pmol/L) (15). Similarly, disorders in which FGF23 is increased, such as X-linked hypophosphatemia and tumor-induced osteomalacia, also result in very low $1,25(OH)_2D$ combined with low phosphate levels. Defects in the VDR, impairing binding and subsequent hormonal activity by $1,25(OH)_2D$ lead to vitamin D-dependent rickets type 2. Characteristically, very high $1,25(OH)_2D$ levels are found in these individuals. Diseases displaying excessive amounts of extrarenal enzymatic formation of $1,25(OH)_2D$, such as sarcoidosis and tuberculosis are also associated with increased levels of $1,25(OH)_2D$ (above 159 pmol/L) (15).

Vitamin D metabolite ratio

The ratio of 25(OH)D to 24,25$(OH)_2$D is useful as a marker of 24α-hydroxylase activity (8). Its activity increases as a means to prevent overproduction of 1,25$(OH)_2$D, for example upon supplementation of vitamin D. It has been revealed to be independent of VDBP and may serve as a better reflector of vitamin D status in patient groups with a larger variety of VDBP concentrations (16). In a recent study by Ginsberg *et al*. for example, it was shown to be more strongly associated with loss of BMD and fracture risk in a cohort of community-dwelling older adults compared to 25(OH)D. Some have suggested the ratio to be a good predictor of adequate vitamin D status after supplementation, but this has not been confirmed by recent publications (17). However, it is significantly increased in, and can therefore be used in the diagnosis of, idiopathic infantile hypercalcemia (IIH) (18, 19). IIH is caused by a mutation in the gene coding for 24α-hydroxylase which impairs the inactivation of 25(OH)D and 1,25$(OH)_2$D and thus leads to overproduction of the active hormone, hypercalcemia and low PTH concentrations (20).

Free and bioavailable total 25-hydroxyvitamin D

Over 85% of 25(OH)D and 1,25$(OH)_2$D is bound to VDBP, while most of the remaining is bound to albumin and only about 0.03% circulates free of any binding protein (21). Some have suggested calculated bioavailable 25(OH)D (not bound to VDBP) or free 25(OH)D (not bound to VDBP or albumin) may be a more relevant biomarker of vitamin D status, especially in those with a different genotype of VDBP (22). However, a number of studies have since determined this not to be the case and showed that both free and bioavailable 25(OH)D only reflect total 25(OH)D and have no or limited added clinical utility (23, 24).This makes sense realizing that 25(OH)D is not the actual hormonally active compound but a prohormone and free 25(OH)D is as a consequence not regulated by feedback loops (14). As long as sufficient amounts of 25(OH)D can be oxidised to form 1,25$(OH)_2$D, the exact pool of free or bioavailable 25(OH)D seems of little importance.

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Measurand

As both 25(OH)D_3 and 25(OH)D_2 may be hydroxylated to an active hormone, measurement of vitamin D status should encompass total 25(OH)D, meaning both the isomers 25(OH)D_3 and 25(OH)D_2. 25(OH)D_2 has an additional double bond compared to 25(OH)D_3 and as a result differs in mass. As mentioned before, we are able to produce 25(OH)D_3 with the help of UVB radiation. Usually, most if not all of the total 25(OH)D is therefore 25(OH)D_3. However, supplementation may be either of the two forms. In the US for instance it is customary to prescribe the D_2 form, while in Europe most formulations contain the D_3 form. This means that the D2/D3 ratio found in clinical practice differs per country. To that end, methods should either distinguish the two components and may sum their concentrations or, if their distinguishing is not possible or wanted, quantify their concentrations together in an equimolar manner. Furthermore, epi-25(OH)D is not part of total 25(OH)D and should therefore ideally not be included in the calculation of total 25(OH)D. Likewise, total 1,25$(OH)_2$D includes isomers 1,25$(OH)_2D_3$ and 1,25$(OH)_2D_2$ and total 24,25$(OH)_2$D includes isomers 24,25$(OH)_2D_2$ and 24,25$(OH)_2D_3$. The D_2 and D_3 isomers differ in their molecular weight. When using a method that does not distinguish between both components, conversion of molar concentrations to weight (e.g. “ng/mL”) is not justifiable as it cannot take the different molecular weights into account (25). Measurement results are therefore to be reported in “nmol/L” in the case of 25(OH)D and 24,25$(OH)_2$D or “pmol/L” for 1,25$(OH)_2$D.

Measurement*Pre analysis*

For 25(OH)D, both automated assays and LC-MS/MS methods mostly do not require a specific sample tube as serum, EDTA or heparin plasma may all be used, but should be checked in the manual before use in case of automated assay and validated in case of LC-MS/MS (26). Samples have been proven very stable for days to months at different storage conditions (-80 degrees to room temperature) and the effects of repeated freeze-thaw cycles is reported to be unsignificant (27-30). For the other two metabolites 1,25$(OH)_2$D and 24,25$(OH)_2$D pre analysis has been less extensively studied unfortunately (31).

Automated assays

Many laboratories rely on automated immunoassays or protein binding assays for determination of total 25(OH)D. The number of tests they process requires a method that is easy in operation and fast. Unlike LC-MS/MS, the automated immunoassay platforms offer such. However, by choosing to focus on easy operation and high throughput, they sacrifice on accuracy. As discussed before, the method used should either distinguish $25(OH)D_3$ and $25(OH)D_2$ and may sum their concentrations or quantify their concentrations together in an equimolar manner. Automated immunoassays are, by virtue of their use of mostly polyclonal antibodies directed towards 25(OH)D with variable affinities that differ for $25(OH)D_3$ and $25(OH)D_2$, unable to truly quantify both components in an equimolar manner (32, 33, 34). The antibodies used show various cross reactivity for $25(OH)D_2$ (35). This is especially problematic in countries where D_2 is frequently described. While cross-reactivity with epi-25(OH)D is not observed in immunoassays, variable cross-reactivity is observed with other more hydroxylated vitamin D metabolites, such as 24,25(OH)D (36). Another pitfall of using an automated immunoassay platform for measurement of 25(OH)D is their lack of accuracy in certain patient groups. Due to varying concentrations of VDBP, and the difficulty the automated assays experience removing vitamin D from its binding proteins, they have difficulty measuring accurately in pregnant women, women on oral contraceptives, patients admitted to the intensive care unit and patients with liver failure (37). Additionally, automated assays struggle with hemodialysis and osteoporotic patients (38, 39). Interestingly, while long-term stability of samples from similar patients measured with LC-MS/MS does not affect the results, a number of the automated immunoassays did show variation of results over time (29).

Similarly, for measurement of $1,25(OH)_2D$ and the Vitamin D metabolite ratio, automated immunoassays lack the ability to distinguish the two isomers (D_3 and D_2) and experience cross reactivity with other metabolites, making these assays less reliable (40).

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On the other side, sample work-up is often diminished completely and tubes, either serum or plasma, may be directly placed on the instrument and no further action is required.

Comparing the costs of running an automated assay and an LC-MS/MS method for vitamin D testing is not easily done as these costs rely on many conditions. In general, machinery and maintenance fee is relatively low in automated assays as the machines are often already in use in clinical laboratories and additional testing will not increase these costs substantially. On the other hand automated assays need relatively expensive reagents that need to be bought from the manufacturers of the specific machines.

LC-MS/MS

During recent years, more labs have turned to LC-MS/MS for their measurement of the vitamin D metabolites as the technique offers superior specificity compared to the automated immunoassays. Importantly, LC-MS/MS does not suffer from cross-reactivity with most analogous vitamin D metabolites that differ little from the desired measurand. Differences in mass can lead to easy separation by the mass spectrometry, or alternatively, these metabolites may be separated by liquid chromatography. Only epi-25(OH)D, with the same molecular mass as 25(OH)D and rather difficult to separate on the LC, is co-measured in many LC-MS/MS methods. Luckily, for most adults, concentrations of epi-25(OH)D are low compared to 25(OH)D and do not often significantly alter total 25(OH)D quantification (41). In infants, greater epi-25(OH)D concentrations may falsely increase total 25(OH)D results when using a LC-MS/MS method not able to separate the epimers (6, 42). The current mass spectrometer methods are sensitive enough to accurately quantify the lower concentrations of $24,25(OH)_2D$ and $1,25(OH)_2D$. Although for $1,25(OH)_2D$ measurements an immunoprecipitation may be helpful (15). Another advantage of LC-MS/MS measurements is the fact that this technique allows quantification of the multiple metabolites at once, thereby supporting studying the relationship between the different vitamin D metabolites, such as those expressed in the Vitamin D metabolite ratio. The technique, however, requires sufficiently trained technicians capable of developing, validating and running the

applications. Today, this may be the biggest hurdle to overcome for LC-MS/MS as a technique to be overall superior to the automated immunoassay, as the quality of the used LC-MS/MS methods seems to differ substantially among laboratories (43). Just as with the automated immunoassays, standardization is still lacking, and may improve the overall quality. This should be feasible, as certified reference material is available for the aforementioned measurands (44). Much effort have been put into the assessment of the commutability and stability of these reference materials (45,46). It again shows the superiority of LC-MS/MS compared to the automated assays. Currently, fully automated LC-MS/MS machines enter the market which are designed to measure 25(OH)D_3 and 25(OH)D_2 separately. This makes it easier for laboratories without the expertise to run LC-MS/MS methods to measure vitamin D. However, these machines are not yet capable of measuring other metabolites in the same run, and up till now studies do not show outstanding method comparisons (47). This might be a result of the compromises a fully automated LC-MS/MS machine has to make on accuracy for easy and fast operation.

Contrary to the automated assays, the costs of running an LC-MS/MS method for vitamin D testing are primarily made up of instrument, maintenance and labor costs, as the machines are expensive and well-trained technicians are required yet chemicals are available at relatively low costs. The costs for personnel totally depend on the degree of automation of processing the samples before putting them on the LC-MS/MS machine.

Conclusion

25(OH)D remains the best indicator of vitamin D status, while only specific conditions may require measurement of the other described metabolites. Whether using an automated immuno- or protein binding assay platform or LC-MS/MS for measurement of vitamin D metabolites, be aware of the intended measurand and the inherent pitfalls of the technique. The automated assays are fast and easily operated, but lack the accuracy to produce accurate total 25(OH)D results. LC-MS/MS has proven excellent at determination of total 25(OH)D and other vitamin D metabolites and further standardization efforts will improve the

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overall quality of LC-MS/MS methods worldwide.. While automated assays are still widely used and may be adequate for a largely healthy population not using D_2, LC-MS/MS allows for vitamin D metabolite profiling, enabling us to study vitamin D metabolism in detail and aids us in more complex cases such as samples containing D_2 or samples from specific patient groups(48, 49). For a concise summary of the current applicability, advantages and disadvantages of the two techniques, see table 1.

Declaration of interest

The authors declare there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Figure 1. Endogenous vitamin D_3 metabolism. When our skin is penetrated by UVB light, 7-dehydrocholesterol is converted to pre-vitamin D. This rapidly isomerizes into vitamin D3, which then enters circulation and binds to vitamin D binding protein (VDBP). Liver enzyme 25α-hydroxylase then hydroxylates vitamin D_3, which gains $25(OH)D_3$. $25(OH)D_3$ can be converted into the active hormone, $1,25(OH)_2D_3$ by 1α-hydroxylase. Alternatively, it may be epimerized to epi-$25(OH)D_3$ or to $24,25(OH)_2D_3$, inactive metabolites with no or substantially lower affinity for 1α-hydroxylase.

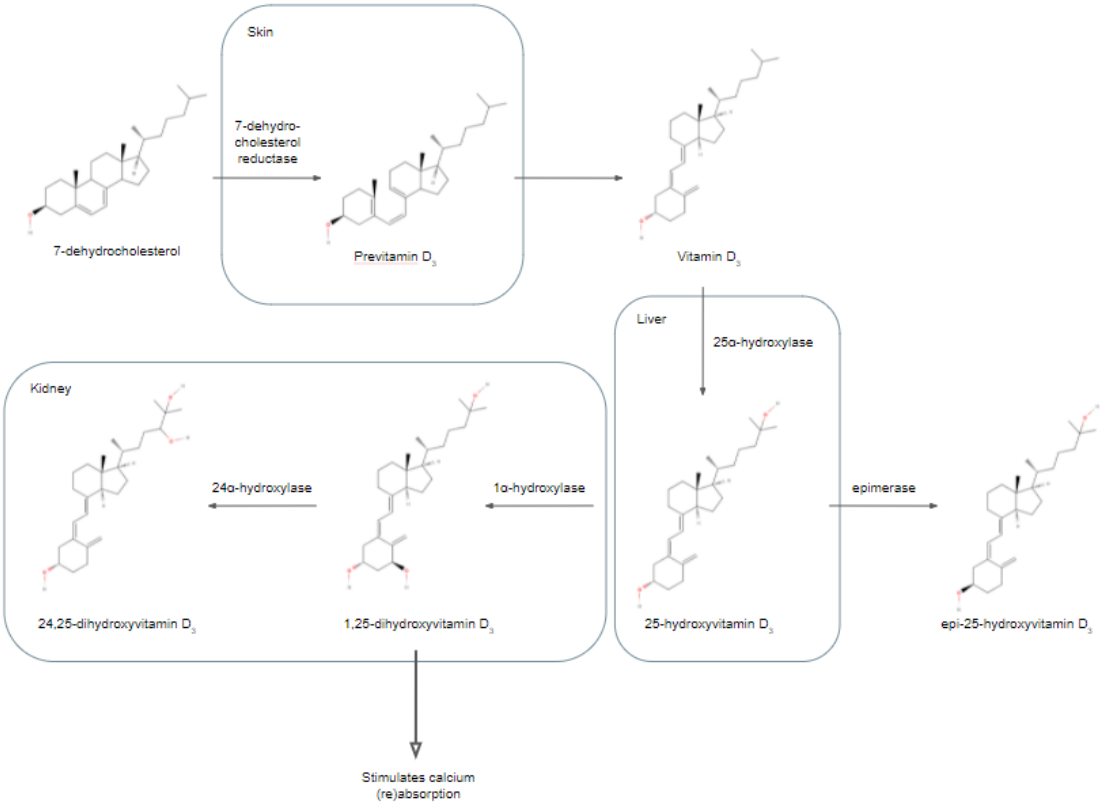

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Table 1. Availability of reference material and reference measurement procedures (RMP) and advantages and disadvantages of immunoassays and LC-MS/MS.

	25(OH)D	**$1,25(OH)_2D$**	**$24,25(OH)_2D$**
NIST standard(s)	SRM 972a, SRM 2969, SRM 2970, SRM 2972a, SRM 2973	None	SRM 972a, SRM 2972a, SRM 2973
RMP	Yes (LC-MS/MS based)	No	Yes (LC-MS/MS based)

	Advantages	**Disadvantages**
Immunoassay	Fast, easy operation, no cross-reactivity with epi-forms	Reduced accuracy in certain patient groups, no distinction between D_2 and D_3 forms, variable cross reactivity with other related metabolites
LC-MS/MS	Specificity, possibility of metabolite profiling	Complexity, difficulty to separate isomers