In this issue: Laboratory investigation of vitamin D and bone metabolism markers

The Journal of the International Federation of Clinical Chemistry and Laboratory Medicine

eJIFCC

Vitamin D and its metabolites: from now and beyond

Etienne Cavalier[1], Jean-Claude Souberbielle[2]

[1] *Department of Clinical Chemistry, University of Liege, CHU de Liège, Belgium*

[2] *Laboratoire d'Explorations fonctionnelles, Hôpital Necker-Enfants malades, Paris, France*

ARTICLE INFO

Corresponding author:
Etienne Cavalier
Department of Clinical Chemistry
University of Liège
4000 Liège
Belgium
E-mail: Etienne.cavalier@chuliege.be

Key words:
25 hydroxyvitamin D,
1,25 dihydroxyvitamin D,
standardization,
Vitamin D Metabolism Ratio (VMR)

ABSTRACT

Total 25-hydroxyvitamin D is currently considered as the most representative metabolite of vitamin D status. There are a multitude of challenges that still deserve to be addressed and despite recent technological advances its determination remains complicated. This current review gives an abbreviated overview of the phases of development that vitamin D metabolite determination has gone through and discusses the difficulties that still require resolving. Furthermore, given the different platforms and methodologies available, the critical issue of standardization and all efforts made as far towards its realization have been discussed. And last but not least, the concepts of 'free' and 'bioavailable' vitamin D along with the 'Vitamin D Metabolism Ratio' have been discussed.

INTRODUCTION

Until recently, 25(OH)-vitamin D (25-OHD) was merely the only vitamin D (VTD) metabolite of interest to explore vitamin D status and metabolism. Unfortunately, the determination of this VTD metabolite, as well as the levels that need to be achieved in healthy or diseased individuals are quite problematic and remain an important matter of debate [1.2].

Recently, other VTD metabolites, like 24,25-OH2D, "bioavailable" or "free" vitamin D, cholecalciferol itself and 1,25-OH2D, have emerged as potential new players to better understand the important vitamin D pathway. In this paper, we provide a brief overview on the issues regarding 25-OHD assays and standardization and we will evoke 24,25(OH)2D and Vitamin D ratio (VMR) as potential metabolites of choice to explore vitamin D deficiency.

25-HYDROXY VITAMIN D DETERMINATION

25-OHD is still currently considered as the most representative metabolite of vitamin D status. Unfortunately, its determination remains complicated despite recent technological advances [3]. The reasons why this metabolite is so complicated to be correctly assessed are multiple. First, 25-OHD assays need to recognize 25-OHD2 and 25-OHD3. Second, 25-OHD is a very hydrophobic molecule that circulates bound to vitamin D binding protein (DBP), albumin (ALB) and lipoproteins and a thorough dissociation of the analyte from its ligands is mandatory prior to measurement. This step is particularly complicated for automated immunoassays where, in contrast to radio-immunoassays, binding-protein or chromatographic assays, organic solvents cannot be used for extraction. Hence, automated immunoassays need alternative releasing agents, which do not always achieve total dissociation of 25-OHD. In particular physiological or pathological conditions such as pregnancy, estrogen therapy or renal failure, automated immunoassays often fail to correctly quantify 25-OHD [4-7]. Third, 25-OHD2 and 25-OHD3 have different affinity constants for the carriers and the dissociation step must be highly efficient to obtain an accurate quantification of both forms. Forth, in-vitro recovery experiments with the molecule give spurious results with immunoassays since it is not clear whether exogenous metabolites bind to all the different carriers in the same proportions as endogenous metabolites. Under-recovery of exogenous 25-OHD has been reported in automated immunoassays [8-9] and even liquid chromatography tandem mass spectrometry (LC-MS/MS) methods [10].

The different methods available for the quantification of 25-OHD use chromatographic separation (HPLC with UV or LC-MS/MS detectors), antibodies or binding-proteins. Binding protein assays have been used in the early eighties and presented clinically acceptable analytical sensitivity and imprecision. They were based on the displacement of H^3-labelled 25-OHD and necessitated a chromatographic purification after organic extraction. These home-made methods were very time-consuming and performed in some reference laboratories only. Hence, they have been superseded by radio-immunoassays (RIA) methods. The first commercially available RIA was based on a method described by Hollis et al. in 1993 [11] and the DiaSorin RIA method has been the most widely used method for both routine diagnostic testing as well as for clinical studies until recently. Traditional 25-OHD cut-offs in use today for vitamin D deficiency (either 20 or 30 ng/ml) have been defined on the basis of studies (and meta-analyses of studies) that predominantly used this assay. However, due to the logarithmic increase in 25-OHD requests observed during the last decade, laboratories have opted for more automated immunoassays and less than 1% of laboratories participating in the DEQAS still use this RIA assays nowadays.

The first automated immunoassay for 25-OHD determination was launched in 2001 by Nichols Diagnostics on the Advantage platform. This assay used a competitive ligand binding technique with acridinium-ester labelled anti-DBP. Nowadays, most of the major in-vitro diagnostic companies have launched their own methods for 25-OHD determination. These methods use a competition design, except the one from Fujirebio on the Lumipulse, which is a non-competitive (sandwich) method based on antimetatype monoclonal antibodies against a hapten–antibody immunocomplex using an ex vivo antibody development system, namely the Autonomously Diversifying Library system, a process which has recently been validated [12]. A large number of studies have evaluated the different automated assays by comparison with RIA, HPLC or, more recently, with LC-MS/MS methods. Conclusions regarding the accuracy of the assays have also been based on the results of large external proficiency testing programs, such as DEQAS or CAP which now use a reference method to measure the samples sent to the participants, allowing a true calculation of the bias. In a recent study coordinated by the Vitamin D Standardization Program (VDSP) group [13], a set of 50 healthy individuals donor samples were analyzed by 15 different laboratories to provide results for total 25-OHD using both immunoassays and LC-MS/MS methods. The results were compared with those obtained by two reference methods, namely the Ghent University and the U.S. National Institute of Standards and Technology (NIST) methods. Results showed that all but 2 LC-MS/MS achieved VDSP criteria of performance (namely CV ≤ 10% and mean bias ≤ 5%), whereas only 50% of immunoassays met the criteria. These results can be regarded as optimistic for immunoassays. First, it is obvious from these results that standard deviation around the bias is much more important for immunoassays than LC-MS/MS. As an example laboratory 2a that used an immunoassay and laboratory 10 used a LC-MS/MS method which both presented an excellent mean bias of -1%. But the standard deviation around this bias was 14% for the immunoassay vs. 5% for the LC-MS/MS method. As a consequence, the LC-MS/MS will have 75% of its value within the 5% boundaries whereas the immunoassay will only have 29%. Second, this study has been performed on serum obtained in healthy donors and not in patients. Indeed, patients with chronic kidney disease, dialysis patients, pregnant women, different ethnic groups, patients in intensive care with fluid shifts present differences in their serum matrix compared to healthy individuals and this can impact the performance of automated 25-OHD immunoassays. Recently, we have shown good clinical concordance between 4 different immunoassays and a VDSP-traceable LC-MS/MS method in healthy subjects. However, significantly poorer agreement with the same LC-MS/MS method has been found in other clinical populations [4, 14].

In the past years, the IFCC has made great efforts to promote standardization of laboratory assays. Indeed, standardization is important to achieve comparable results across different methods and manufacturers. For 25-OHD assays, clinical cut-offs are generally used as target values, and applying common cut-offs on results generated with poorly standardized assays will inevitably lead to inconsistent patient classification and inappropriate therapeutic decisions. Hence, in 2010, the Vitamin D Standardization Program (VDSP) was established to improve the standardisation of 25-OHD assays. The aim of VDSP is that 25-OHD measurements are accurate and comparable over time, location, and laboratory procedure to the values obtained using reference measurement procedures (RMPs) developed at the NIST [15] and Ghent University [16]. As mentioned earlier, a method is considered as standardized if the CV is <10% and the bias <5% [17]. Each candidate receives a set of

10 samples 4 times a year and has to run these samples in duplicate on 2 consecutive days. In January 2018, 27 methods, coming either from IVD companies or clinical laboratories were considered as standardized against the RMPs. However, the proportion of the 40 samples that met the bias criterion (<5%) in 2017 was quite different from one method to the other and ranged from 23 to 85%, with LC-MS/MS methods presenting better results than immunoassays. The list of these standardized methods can be found on the CDC website (http://www.cdc.gov/labstandards/pdf/hs/CDC_Certified_Vitamin_D_Procedures.pdf).

Although substantial progress has been made, a range of important issues like standardization of 25-OHD2 and 24,25-(OH)2D as well as improvement of (immuno)assays performance on samples from diseased patients or subjects from different ethnic groups still needs to be achieved. It may thus be tempting to think that immunoassays are outdated and that LC-MS/MS should replace these methods. There are clear limitations to this simplistic view. Indeed, performing a LC-MS/MS is complex and needs experienced and very well trained people. Notably, extensive validation of the LC-MS/MS and sample preparation are of extreme importance. To run a LC-MS/MS is much more complicated than "crash the proteins, inject and obtain the results in 2 minutes". A detailed review on how complex running a LC-MS is out of the scope of this present paper, but can be found in a previous report [18]. Finally, laboratories that run LC-MS/MS do not run "the" reference method, even if their method is certified by the VDSP. As an illustration of this assertion, one can see that some VDSP-certified LC-MS/MS methods present a percentage of samples out of the bias criterion that is lower than immunoassays and much lower than other LC-MS/MS. Also DEQAS results show that LC-MS/MS methods present CVs that are as high as immunoassays.

24,25-(OH)2D DETERMINATION AND THE VITAMIN D METABOLITE RATIO

One advantage of LC-MS/MS methods over immunoassays is the possibility to simultaneously quantify 25-OHD and 24,25-(OH)2D allowing to calculate the 25-OHD/24,25-(OH)2D ratio, also known as the Vitamin D Metabolism Ratio (VMR). Indeed, some light has recently been shed on the potential interest of this vitamin D metabolite to better reflect vitamin D deficiency [19]. In summary, CYP24A1, the enzyme allowing the degradation of 25-OHD and 1,25-(OH)2D into 24,25-(OH)2D and 1,24,25-(OH)3D sees its expression increased when there is an increased binding and activation of the VDR in response to 1,25-(OH)2D [20]. Hence 24,25-(OH)2D concentration may thus reflect VDR activity which is not really the case with 25-OHD. It has recently been demonstrated that lower 24,25-(OH)2D concentration and lower VMR were associated with increased hip fracture risk in community-living older men and women, whereas 25-OHD was not associated with hip fracture risk. Another point of interest with 24,25-(OH)2D and VMR is that, although concentrations of 25-OHD and 24,25-(OH)2D strongly correlate with each other and are both lower in black Americans than in whites, blacks and whites have equivalent median VMR values [21]. In CKD patients, Bosworth et al have shown that 24,25-(OH)2D was better associated with PTH than 25-OHD or 1,25-(OH)2D [22]. These findings are of great interest but still need to be confirmed by other studies. On the other hand, it is clearly demonstrated that biallelic mutations in CYP24A1 led to idiopathic infantile hypercalcemia [23], a phenotype characterized by profound hypercalcemia, suppressed intact parathyroid hormone, hypercalciuria and nephrocalcinosis. Many heterozygous mutations of CYPA24A1 have recently been described [24]. If they are associated with a less dramatic phenotype than homozygous mutations, patients suffering from these mutations

often present with hypercalcemia, suppressed PTH and renal stones [25]. Hence, in patients presenting with a non-parathyroid hypercalcemia (without evident clinical cause), CYP24A1 mutations should be investigated and simultaneous 24,25-(OH)2D and VMR ratio should be measured. A ratio higher than 50, or even 80 should lead to a genetic research of a CYP24A1 mutation. Again, this measurement should be standardized. Fortunately, one candidate reference measurement procedure (RMP) has been published [26] and NIST standard reference material (SRM) 2972a includes 4 standards with certified values (unfortunately, these 4 values are very close to each other) [27]. DEQAS data report that about 10 laboratories provide 24,25-(OH)2D results. These data show quite a large variability, which can partially be attributed to the low concentration of the analyte, but also to the lack of ongoing standardization program. This latter will be (probably) even more important than the 25-OHD itself since small variations in 24,25-(OH)2D have a dramatic impact on the VMR.

CONCLUSION

The assessment of vitamin D status is a changing landscape [19]. Although 25-OHD is still recommended as the marker of choice by virtually all scientific bodies growing evidence indicates significant limitations that hamper the utility of this analyte in clinical practice. Issues related to the use of 25-OHD include analytical aspects and the interpretation of results. While in normal individuals the agreement of results generated with automated assays is improving, comparability of results in distinct populations, such as children, pregnant women, hemodialysis patients or intensive care patients, remains problematic. The relationships between 25-OHD and various clinical indices are also rather weak and not consistent across races. Recent studies have provided new insights in physiological and analytical aspects of vitamin D that may change the way how we will assess vitamin D status in the future. The VMR (25-OHD/24,25-OH2D ratio), but also 'free' and 'bioavailable' vitamin D are all interesting markers that have expanded our knowledge about vitamin D metabolism and some of these analytes may now be considered for routine use (at least in specialized centers).

REFERENCES

1. Ross AC, Manson JE, Abrams SA, et al. (2011) The 2011 report on dietary reference intakes for calcium and vitamin D from the Institute of Medicine: what clinicians need to know. J Clin Endocrinol Metab 96:53–58.

2. Holick MF, Binkley NC, Bischoff-Ferrari HA, et al. (2012) Guidelines for preventing and treating vitamin D deficiency and insufficiency revisited. J Clin Endocrinol Metab 97:1153–1158.

3. Hollis BW (2004) The determination of circulating 25-hydroxyvitamin D: No easy task. J Clin Endocrinol Metab 89:3149–3151.

4. Moreau E, Bächer S, Mery S, et al. (2015) Performance characteristics of the VIDAS® 25-OH Vitamin D Total assay – comparison with four immunoassays and two liquid chromatography-tandem mass spectrometry methods in a multicentric study. Clin Chem Lab Med.

5. Depreter B, Heijboer AC, Langlois MR (2013) Accuracy of three automated 25-hydroxyvitamin D assays in hemodialysis patients. Clin Chim Acta 415:255–260.

6. Heijboer AC, Blankenstein M a., Kema IP, Buijs MM (2012) Accuracy of 6 routine 25-hydroxyvitamin D assays: Influence of vitamin D binding protein concentration. Clin Chem 58:543–548.

7. Cavalier E, Lukas P, Crine Y, et al. (2014) Evaluation of automated immunoassays for 25(OH)-vitamin D determination in different critical populations before and after standardization of the assays. Clin Chim Acta 431:60–65.

8. Carter GD, Jones JC, Berry JL (2007) The anomalous behaviour of exogenous 25-hydroxyvitamin D in competitive binding assays. J Steroid Biochem Mol Biol 103:480–482.

9. Horst RL (2010) Exogenous versus endogenous recovery of 25-hydroxyvitamins D2 and D3 in human samples using high-performance liquid chromatography and the DiaSorin LIAISON Total-D Assay. J Steroid Biochem Mol Biol 121:180–182.

10. Lankes U, Elder P a., Lewis JG, George P (2015) Differential extraction of endogenous and exogenous 25-OH-vitamin D from serum makes the accurate quantification

in liquid chromatography-tandem mass spectrometry assays challenging. Ann Clin Biochem An Int J Biochem Lab Med 52:151–160.

11. Hollis BW, Kamerud JQ, Selvaag SR, et al. (1993) Determination of vitamin D status by radioimmunoassay with an 125I-labeled tracer. Clin Chem 39:529–533.

12. Omi K, Ando T, Sakyu T, et al. (2015) Noncompetitive Immunoassay Detection System for Haptens on the Basis of Antimetatype Antibodies. Clin Chem 61:627–635.

13. Wise SA, Phinney KW, Tai SSC, et al. (2017) Baseline Assessment of 25-Hydroxyvitamin D Assay Performance: A Vitamin D Standardization Program (VDSP) Interlaboratory Comparison Study. J AOAC Int 100:1244–1252.

14. Cavalier E, Lukas P, Bekaert A-C, et al. (2016) Analytical and clinical evaluation of the new Fujirebio Lumipulse®G non-competitive assay for 25(OH)-vitamin D and three immunoassays for 25(OH)D in healthy subjects, osteoporotic patients, third trimester pregnant women, healthy African subjects, hemodiadialyzed and intensive care patients. Clin Chem Lab Med 54(8):1347-55.

15. Tai SS-C, Bedner M, Phinney KW (2010) Development of a candidate reference measurement procedure for the determination of 25-hydroxyvitamin D3 and 25-hydroxyvitamin D2 in human serum using isotope-dilution liquid chromatography-tandem mass spectrometry. Anal Chem 82:1942–1948.

16. Stepman HCM, Vanderroost A, Van Uytfanghe K, Thienpont LM (2011) Candidate Reference Measurement Procedures for Serum 25-Hydroxyvitamin D3 and 25-Hydroxyvitamin D2 by Using Isotope-Dilution Liquid Chromatography-Tandem Mass Spectrometry. Clin Chem 57:441–448.

17. Stöckl D, Sluss PM, Thienpont LM (2009) Specifications for trueness and precision of a reference measurement system for serum/plasma 25-hydroxyvitamin D analysis. Clin Chim Acta 408:8–13.

18. Herrmann M, Farrell C-JL, Pusceddu I, et al. (2017) Assessment of vitamin D status – a changing landscape. Clin Chem Lab Med 55(1):3-26

19. Ginsberg C, Katz R, de Boer IH, et al. (2018) The 24,25 to 25-hydroxyvitamin D ratio and fracture risk in older adults: The cardiovascular health study. Bone 107:124-130.

20. Tryfonidou M a, Oosterlaken-Dijksterhuis M a, Mol J a, et al. (2003) 24-Hydroxylase: potential key regulator in hypervitaminosis D3 in growing dogs. Am J Physiol Endocrinol Metab 284:E505-13.

21. Berg AH, Powe CE, Evans MK, et al. (2015) 24,25-Dihydroxyvitamin D3 and Vitamin D Status of Community-Dwelling Black and White Americans. Clin Chem 61(6):877-84.

22. Bosworth CR, Levin G, Robinson-cohen C, et al. (2012) The serum 24,25-dihydroxyvitamin D concentration, a marker of vitamin D catabolism, is reduced in chronic kidney disease. Kidney Int 82:693–700.

23. Schlingmann KP, Kaufmann M, Weber S, et al. (2011) Mutations in CYP24A1 and Idiopathic Infantile Hypercalcemia. N Engl J Med 365:410–421.

24. Molin A, Baudoin R, Kaufmann M, et al. (2015) CYP24A1 mutations in a cohort of hypercalcemic patients: Evidence for a recessive trait. J Clin Endocrinol Metab 100:E1343–E1352.

25. Ferraro PM, Minucci A, Primiano A, et al. (2016) A novel CYP24A1 genotype associated to a clinical picture of hypercalcemia, nephrolithiasis and low bone mass. Urolithiasis 25:1–4.

26. Tai SS-C, Nelson M a. (2015) Candidate Reference Measurement Procedure for the Determination of (24 *R*),25-Dihydroxyvitamin D3 in Human Serum Using Isotope-Dilution Liquid Chromatography–Tandem Mass Spectrometry. Anal Chem 87:7964–7970.

27. Wise SA, Tai SSC, Burdette CQ, et al. (2017) Role of the national institute of standards and technology (NIST) in support of the Vitamin D initiative of the national institutes of health, office of dietary supplements. J AOAC Int 100:1260–1276.