

Analytical considerations for the biochemical assessment of vitamin D status

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Abstract: The most widely used and clinically accepted biochemical marker for assessing vitamin D status is the total serum 25-hydroxyvitamin D [25(OH)D] concentration. Despite the analysis of 25(OH)D dating back to the early 1970s, modern analytical techniques still exhibit significant interassay variability due to varying concentrations of other related vitamin D metabolites and sample-to-sample matrix differences. It is important for clinicians requesting 25(OH)D analyses to understand these issues and limitations, and where necessary to confront laboratories for details of analytical methods used. The availability of reference measurement procedures for 25(OH)D based on liquid chromatography and tandem mass spectrometry, whilst not intended for routine clinical sample analysis, should be utilized to improve assay harmonization and reduce interlaboratory variability. Laboratories should also be forthcoming with details of subscriptions to external quality assessment schemes and assay traceability. As well as discussing the reasons for ongoing assay variability for 25(OH)D, this short review will also briefly discuss other assays related to the assessment of vitamin D status, including parathyroid hormone, 24,25-dihydroxyvitamin D, 1,25-dihydroxyvitamin D and vitamin D binding proteins.

Keywords: Vitamin D, vitamin D status, 25-hydroxyvitamin D, immunoassay, LC-MS/MS, 1,25-dihydroxyvitamin D, 24,25-dihydroxyvitamin D, vitamin D binding protein

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Introduction

‘Vitamin D status’ is used to define whether an individual is vitamin D deficient, sufficient, or intoxicated.¹ The most widely used and clinically accepted biochemical marker for assessing vitamin D status is the total serum 25-hydroxyvitamin D [25(OH)D] concentration.² This is due to the relatively long circulating half life of the compounds and the fact that these compounds are not subject to tight homeostatic control.^{3–5}

Defining vitamin D status according to ‘threshold’ serum 25(OH)D concentrations is however a topic of considerable ongoing debate. The Institute of Medicine guidelines, devised using bone health outcomes to suggest dietary intake, suggest that individuals are at risk of vitamin D deficiency at 25(OH)D concentrations below 30 nmol/liter (12 µg/liter), some individuals are at risk of inadequacy at serum 25(OH)D concentrations between 30 and 50 nmol/liter

(12–20 µg/liter), and almost all individuals are sufficient at concentrations of 50 nmol/liter (20 µg/liter) or greater.⁶ The Endocrine Society guidelines, in contrast, concluded that 50 nmol/liter should be used as a ‘cut-off’ for vitamin D deficiency and that to maximize the effect of vitamin D on calcium, bone, and muscle metabolism, serum 25(OH)D should exceed 75 nmol/liter (30 µg/liter).^{7,8} Published guidelines issued following an international meeting of vitamin D experts held in Warsaw, Poland in 2012 concluded that the target concentration for 25(OH)D should be 30–50 ng/ml, or even up to 100 ng/ml.⁹ More recently, and again in contrast to these suggestions, the clinical value of very high 25(OH)D concentrations has been questioned.¹⁰

In some situations there is no doubt that analysis of 25(OH)D is useful to confirm clinical observations, for instance in severe vitamin D deficiency. However, as will be discussed, the analysis of

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25(OH)D is not straightforward. It is likely that variability in the analysis of 25(OH)D contributes to the ongoing debate regarding threshold 25(OH)D 'reference ranges'. It should be remembered that the vitamin D metabolic pathway is a highly complex and dynamic system involving a number of structurally similar compounds.¹¹ The analysis of compounds other than 25(OH)D in assessing true vitamin D status can be important in some clinical situations and should ideally be considered alongside interactions with other calcitropic hormones such as parathyroid hormone (PTH), as well as plasma transporter proteins such as vitamin D binding proteins (VDBPs).

There are two considerations which clinicians requesting the assessment of vitamin D status in their patients should be aware of and which will be discussed in this short review. First, causes of variability in the analytical approaches to the measurement of 25(OH)D, which highlights the importance of ongoing work towards harmonization of 25(OH)D testing.¹² Second, the possible value of measuring additional biomarkers to give a more complete picture of vitamin D status and the use of modern analytical approaches to address the analysis of some of these compounds.^{13,14}

Analysis of 25-hydroxyvitamins D

It is important to realize the limitations of 25(OH)D assays and to understand how they are related to the physiology of vitamin D metabolism. Every analytical platform used for 25(OH)D analysis has both advantages and disadvantages that give rise to interassay variability and these can be largely attributed to two important factors. First, there are a number of structurally related hydrophobic compounds in the circulation which may interfere with measurement; and second, vitamin D metabolites circulate in the plasma tightly bound to VDBPs.¹⁵

Analysis of 25(OH)D dates back to the early 1970s and the use of competitive protein binding assays,¹⁶ but current analytical techniques tend to be either immunoassay based, or chromatography based with ultraviolet (UV) or mass spectrometric detection. Immunoassay-based techniques are inherently sensitive, often requiring low sample volumes compared with chromatographic methods. A major attraction to high-throughput clinical chemistry laboratories is that these assays are easily integrated into fully automated, random-access

laboratory track systems, thus allowing rapid analysis times. However, assay selectivity due to cross reactivity with different vitamin D metabolites is a significant limitation. This is simply illustrated by considering the cross reactivity with 25-hydroxyvitamin D₂ (25(OH)D₂, calcidiol, ergocalciferol) and 25-hydroxyvitamin D₃ (25(OH)D₃, calcitriol), and two immunoassay-based methods: method A which is demonstrated to cross react equally for the two compounds; and method B which only partially cross reacts with 25(OH)D₂. For a patient supplemented with vitamin D₂, the presence of 25(OH)D₂ in the circulation will mean, purely due to an analytical artefact, that the reported result for total 25(OH)D₂ will be lower using method B. Though often at lower concentrations in the circulation, the same rationale can be applied to other vitamin D metabolites, for example 24,25-dihydroxyvitamin D [24,25(OH)₂D], an inactivated product of 25(OH)D₃ produced and excreted by the kidneys and likely to cross react to different degrees in immunoassays from different vendors.

A further limitation of immunoassay-based analytical platforms relates to VDBPs. To measure the total 25(OH)D, the analytes must first be liberated from the binding proteins. The methods used to achieve this in automated methods are often proprietary. Related to this, it has been shown that variation in VDBP concentration (e.g. due to pregnancy or for patients on dialysis) has an effect on some automated immunoassays for the measurement of 25(OH)D.¹⁷

Chromatography-based techniques have been used for a number of years for the analysis of vitamin D metabolites. Indeed, solvent extraction and chromatographic fractionation was used in the very first analytical method for 25(OH)D, already mentioned in the 1970s.¹⁶ The independent analysis of vitamin D metabolites using specific detection methods offers a number of advantages. Most obviously, following extraction to disrupt protein binding, 25(OH)D₂ can be resolved chromatographically from 25(OH)D₃, and thus independently calibrated and quantified. Early chromatographic methods were based on liquid chromatography (LC) with UV detection,¹⁸ but recently there has been a dramatic shift towards the use of LC coupled to tandem mass spectrometry (LC-MS/MS). Selectivity in LC-UV methods is achieved *via* chromatographic resolution of the individual metabolites, since the detection system is relatively nonspecific. With MS, in addition to chromatographic separation,

vitamin D metabolites are selectively detected based on their mass-to-charge ratio (m/z) values. Furthermore, and crucially for quantitative analyses such as 25(OH)D, MS allows the inclusion of stable isotope-labelled internal standards.

Despite obvious advantages, LC-MS/MS assays for 25(OH)D are not always without fault and are certainly not fool proof. Although there has been substantial progress in automation of LC-MS/MS technology in the past few years,¹⁹ complete 'primary sample-to-result' LC-MS/MS workflows are not yet automated to the extent of immunoassays. The complexity of LC-MS/MS instrumentation, and the fact that methods are not 'locked down' as with immunoassay kits, means there are a number of instrumentation parameters that will vary between laboratories and can potentially cause interlaboratory variability.²⁰ Having samples measured by a routine LC-MS/MS method does not guarantee an accurate result, though the flexibility of LC-MS/MS as an analytical platform makes this approach the current 'gold standard'. Take the example of 3-*epi*-25-hydroxyvitamin D₃ (3-*epi*-25(OH)D₃). This vitamin D metabolite was first reported to accumulate in samples from neonates,²¹ though more recent literature suggests it can also be present in samples from adults,²² often at relatively low concentrations or low proportions of the total 25(OH)D.²³ The source of the 3-*epi* isomer and its biochemical functions remain largely unclear²⁴ and its relevance to assessing vitamin D status in the context of this review remains primarily as an analytical artefact in LC-MS/MS methods. This metabolite differs only from 25(OH)D₃ by the stereochemistry of the hydroxyl group at position 3 (i.e. they are entirely isobaric). Immunoassays do not tend to cross react with this metabolite,¹⁵ but since MS is an achiral technique, it is unable to differentiate these two metabolites unless they are chromatographically resolved from one another. With modern instrumentation, the latter is easily achieved without requiring significantly increased analysis times.²⁵ Furthermore, once chromatographically separated (and with suitable analytical standards to calibrate the assay), it is possible to prospectively quantify the 3-*epi* isomer in samples from different patient groups to assess possible impact on vitamin D status and to answer some of the outstanding questions regarding the physiology of this metabolite. A number of groups have already investigated this and have found typically low, but variable concentrations of the 3-*epi* isomer relative to 25(OH)D.²⁴

Reference method procedures, assay harmonization and assay standardization

Harmonization of 25(OH)D testing has been a challenge for many years. The Vitamin D External Quality Assessment Scheme (DEQAS) was established in 1989 in response to concerns over the poor performance and large variability between 25(OH)D assays,^{26,27} and has dramatically reduced the variability between participating laboratories since its inception.

More recently, efforts to harmonize 25(OH)D analysis have been significantly aided by the availability of standard reference materials (SRMs) and by the development of reference method procedures (RMPs). The most recent SRM (SRM 972a) from the National Institute of Standards and Technology (NIST), produced in collaboration with the National Institutes of Health Office of Dietary Supplements, contains four human serum-based solutions containing different concentration levels of vitamin D metabolites, including 25(OH)D₂, 25(OH)D₃, 3-*epi*-25(OH)D₃, and 24,25-dihydroxyvitamin D₃ [24,25(OH)₂D₃]. These SRMs are commercially available and are designed for use by clinical laboratories for method validation. Each level SRM is supplied with certified reference values assigned using the NIST RMP.^{28,29} Subsequent methods by the University of Ghent and the Center for Diseases Control (CDC) have since been validated and accredited to ISO 15193 and JCTLM guidelines.^{30,31} All three methods are based on isotope-dilution LC-MS/MS, including 3-*epi* isomer resolution, and all three groups are involved in the ongoing efforts to harmonize 25(OH)D testing as organized through the Vitamin D Standardization Programme. However, RMPs are expensive and time consuming, and thus not suited to the environment of a high-throughput clinical diagnostic laboratory for routine 25(OH)D analysis. Instead, these RMPs are invaluable for laboratories with regards to assigning reference values to serum-based samples to assess individual assay performance. All DEQAS samples are now assigned concentrations using the NIST RMP as well as the all-laboratory trimmed mean calculated from the returned results. All College of American Pathologists' external quality control (EQA) samples are assigned concentrations using the CDC RMP. The CDC also offers a standardization certification programme which laboratories may choose to subscribe to.³² Laboratories should ensure that information regarding active participation in EQA schemes (DEQAS) and

method traceability to SRMs and/or a RMP are made available to clinicians wherever possible, and clinicians should ask the laboratory for a statement on assay traceability if in any doubt. Laboratories should ideally provide evidence for cited reference ranges.

Value in the analysis of additional biomarkers

24R,25-Dihydroxyvitamin D

The utility of measuring 24,25(OH)₂D for assessment of vitamin D status remains an issue of debate. 24,25(OH)₂D is the most abundant product of catabolism by 25-hydroxyvitamin D-24-hydroxylase (CYP24A1) of 25(OH)D. Mean circulating concentrations of 24,25(OH)₂D are approximately 10% those of 25(OH)D,³³ but this proportion varies significantly (2–20%) between individuals, especially when 25(OH)D is low.³⁴ Studies have suggested that when 24,25(OH)₂D is detectable, there is good correlation between serum 24,25(OH)₂D and 25(OH)D concentrations. However, at low 25(OH)D concentrations (<25 nmol/liter, 10 µg/liter), 24,25(OH)₂D is often below analytical limits of detection.²⁵

Elevation in the ratio of serum 25(OH)D to 24,25(OH)₂D is clinically useful to diagnose very rare cases of hypercalcaemia due to loss-of-function CYP24A1 mutations.^{25,35} The ratio may also be useful as an adjunct to 25(OH)D for assessing vitamin D deficiency, though this conclusion requires some further work and is of course problematic when 24,25(OH)₂D is undetectable (i.e. at low 25(OH)D concentrations). Two studies have shown that as the 25(OH)D to 24,25(OH)₂D ratio increases, the response of 25(OH)D₃ following supplementation with vitamin D₃ decreases.^{33,36} Clinical use of the ratio in patients with chronic kidney disease (CKD) may also be an issue, since it has been shown that serum 24,25(OH)₂D concentrations are universally low in such patient groups, irrespective of the corresponding 25(OH)D concentration.³⁴

From an analytical perspective, the analysis of 24,25(OH)₂D is currently a specialist test available in only a few referral laboratories and is typically carried out using LC-MS/MS. Often, to increase analytical sensitivity, the compounds require chemical derivatization prior to detection,²⁵ though this is not always the case with newer, more sensitive LC-MS/MS instruments.²⁹

In recognition of the possibility that 24,25(OH)₂D may be a useful additional biomarker, and in response to interest in its measurement from a number of groups, a candidate RMP for 24,25(OH)₂D has recently been developed by NIST.²⁹ There is also some evidence to suggest that 24,25(OH)₂D cross reacts in 25(OH)D immunoassays and so could be problematic for 25(OH)D measurement in patients in whom 24,25(OH)₂D may be elevated.³³

1,25-Dihydroxyvitamin D

It is incorrect to assume that since 1,25-dihydroxyvitamin D [1,25(OH)₂D] is the active hormonal form of vitamin D it should be the marker best used to assess vitamin D status. Rather than reflect the body store of vitamin D, 'normal' serum 1,25(OH)₂D concentrations are 1000-fold lower than 25(OH)D (typical reference ranges from ~6 to 30 pmol/liter, ~15–75 pg/ml) and are under tight homeostatic control. Reflective of this is that serum 1,25(OH)₂D is frequently normalized to within the reference range, or may even be elevated in subjects with vitamin D deficiency associated with low 25(OH)D due to secondary hyperparathyroidism.^{7,37} Only at very low 25(OH)D concentrations, below ~10 nmol/liter (4 µg/liter), is 1,25(OH)₂D found to decrease and this is presumed to be due to a lack of substrate.³⁸ In these patients, both 25(OH)D and 1,25(OH)₂D have been shown to normalize following supplementation.³⁹

There are, however, some clinical circumstances which may warrant serum 1,25(OH)₂D investigation. Although not necessarily an indication to request measurement, it should be noted that in patients with CKD who are not supplemented with calcitriol, 1,25(OH)₂D concentrations tend to decrease following the fall in glomerular filtration rate due to impaired 1-α-hydroxylase (CYP27B1) production.³⁹ A less frequent cause for low serum 1,25(OH)₂D is vitamin D hydroxylation-deficient rickets type 1A (VDDR I), caused by rare autosomal recessive mutations in the CYP27B1 gene. Increases in serum 1,25(OH)₂D result from extrarenal 1-α hydroxylation (e.g. in sarcoidosis or granulomatous disease)⁴⁰ or due to hereditary vitamin D hydroxylation-deficient rickets type 2A (VDDR II), caused by mutations in the vitamin D receptor gene.

In vitamin D toxicity, 1,25(OH)₂D has been shown to be normal or even decreased. The

toxicity has been shown using rodent models not to be attributed to $1,25(\text{OH})_2\text{D}$.³ Biochemically, decreased $1,25(\text{OH})_2\text{D}$ in vitamin D toxicity mirrors the suppression of PTH in hypercalcaemia.⁴¹ However, this observation raises an interesting analytical consideration with regards the measurement of $1,25(\text{OH})_2\text{D}$. The analysis of $1,25(\text{OH})_2\text{D}$ is analytically challenging due to the very low concentrations present and the fact that potential interferences are present at significantly greater concentrations. It has been demonstrated using a spiking study to mimic $25(\text{OH})\text{D}$ concentrations in cases of vitamin D toxicity that serum $1,25(\text{OH})_2\text{D}$ concentrations are artificially elevated due to $25(\text{OH})\text{D}$ interference;⁴¹ this is a much more likely explanation for increased $1,25(\text{OH})_2\text{D}$ in such cases than possible rare genetic mutations (e.g. in CYP24A1). Interestingly, interference was observed for both a commonly used radioimmunoassay and an LC-MS/MS method for $1,25(\text{OH})_2\text{D}$, although the effect was much less pronounced in the latter method. As with $24,25(\text{OH})_2\text{D}$ by LC-MS/MS, analytical sensitivity can be improved by chemical derivatization prior to detection, but again this test is highly specialized and only available in a few laboratories. DEQAS do offer a proficiency testing scheme for $1,25(\text{OH})_2\text{D}$ which should be used wherever possible to ensure results are accurate. When serum $1,25(\text{OH})_2\text{D}$ analysis is indicated to aid diagnosis, although immunoassays are inherently sensitive and will typically require smaller sample volumes per assay, LC-MS/MS is arguably the preferred analytical method. This is especially so in cases of suspected vitamin D toxicity when concentrations of interferences may be raised.

Vitamin D binding proteins

VDBP belongs to the albumin gene family.⁴² There are three major genetic isoforms of serum VDBP in humans: Gc2, Gc1f, and Gc1s,⁴³ which result from polymorphisms of the GC gene. The relevance of VDBP isoforms to vitamin D status has received much literature attention recently. The allele frequencies of the three major VDBP isoforms are strongly linked to geographical distribution. The Gc-1f allele frequency is markedly lower in white populations than in black American and black African populations. White people have a significantly higher Gc-2 allele frequency.⁴⁴

In 2013, Powe and coworkers reported data from a large cohort of black and white community-dwelling American adults, in whom VDBP was

measured by immunoassay and vitamin D status (as 'bioavailable vitamin D') was assessed by calculation.⁴⁵ The key findings of this study were that black Americans had lower $25(\text{OH})\text{D}$ than white Americans, but since black Americans also had lower concentrations of VDBP, the calculated bioavailable vitamin D was a better marker of vitamin D status than $25(\text{OH})\text{D}$. More recently, however, it has been demonstrated that monoclonal immunoassays, including that used for the Powe study, are subject to isoform-specific differences in cross reactivity. When measured by LC-MS/MS, VDBP concentrations do not vary by race/VDBP genotype⁴⁶ and so previous conclusions may be misleading. Further studies are required using either polyclonal immunoassays or LC-MS/MS-based methods which do not show isoform-specific analytical bias to understand the importance of different VDBP isoforms and VDBP concentrations in the assessment of vitamin D status.⁴⁷

Recently, an enzyme-linked immunosorbent assay method to directly measure free $25(\text{OH})\text{D}$ has become commercially available. To date, studies have demonstrated strong correlation between free $25(\text{OH})\text{D}$ and total $25(\text{OH})\text{D}$ concentrations, irrespective of race and GC genotype.⁴⁸

Parathyroid hormone

Whilst $25(\text{OH})\text{D}$ measurement in isolation is currently the most commonly used biochemical marker to clinically define vitamin D deficiency/sufficiency, serum PTH concentrations are inversely correlated with $25(\text{OH})\text{D}$ concentrations, and it is this PTH- $25(\text{OH})\text{D}$ relationship which forms the basis of many suggested cutoff concentrations to make these clinical decisions. Generally, serum PTH is raised in vitamin D deficiency and interrogation of the $25(\text{OH})\text{D}$ concentration at which serum PTH levels plateau is used to define vitamin D sufficiency. However, this is not always the case, since not all patients with hypovitaminosis D will develop secondary hyperparathyroidism.⁴⁹

In terms of analytical considerations, whilst the measurement of calcium as an additional marker to aid PTH interpretation is relatively straightforward, the analysis of PTH (as the 'intact' 84-amino acid peptide) is complex. This is especially relevant for patients with CKD in whom PTH fragments can accumulate and cause assay interference,⁵⁰ even when analysed using LC-MS/MS.⁵¹ As with $25(\text{OH})\text{D}$, there is a clinical need

for the harmonization of PTH testing,⁵² but this requires a reference method, which is unfortunately not available at present. Until such time, clinicians should be aware of the limitations of PTH analysis when requesting testing in patients with CKD.

Conclusion

Assessing vitamin D status is clinically important, and directly impacts clinical decisions to treat or not treat patients. It remains the case that, either in isolation, or in combination with other well established biomarkers such as PTH, serum 25(OH)D is the most useful biomarker available. However, it is clear that the analysis of 25(OH)D is challenging and harmonization of testing between laboratories is still problematic. Clinicians should be aware of these pitfalls and if the information is not readily available, should ask their local laboratory for reassurance that the assay used is traceable. Participation in an EQA scheme such as DEQAS should be highly recommended. The 'gold-standard' 25(OH)D analysis uses LC-MS/MS, but these RMPs are often very different from those carried out in routine, high-throughput laboratories. These latter methods are designed to increase throughput and in larger laboratories are now at least semiautomated. Again, these methods should be fully traceable to a higher order reference standard.

Analysis of additional biomarkers is useful in some rare clinical circumstances, in severe vitamin D deficiency and in cases of suspected vitamin D toxicity (as determined by serum 25(OH)D), and tests are available in a few specialist centres for this purpose. As with 25(OH)D, LC-MS/MS is emerging as the method of choice for these metabolite assays. More recent developments regarding the analysis of VDBP isoforms, and direct analysis of free 25(OH)D, do require some further work, but are already providing useful insights into population-wide (or genotype-specific) differences.

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Conflict of interest statement

The authors declare that there is no conflict of interest.

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