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Determination of vitamin D and its metabolites

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The demand for analysis of 25-hydroxyvitamin D has increased dramatically throughout the world over the past decade. As a consequence, a number of new automated assays have been introduced for 25-hydroxyvitamin D measurement. Automated assays have shown variable ability to meet the technical challenges associated with 25-hydroxyvitamin D measurement. Assays are able to meet performance goals for precision at high concentrations but fail to do so at low concentrations of 25-hydroxyvitamin D. The overall accuracy of automated methods has improved over recent years and generally shows good overall agreement with reference methods; however, discrepancies persist for individual samples. Liquid chromatography-tandem mass spectrometry is used by some routine laboratories for 25-hydroxyvitamin D analysis but its widespread use is hampered by limited sample throughput. 1,25-Dihydroxyvitamin D is an important analyte in specific clinical situations, which remains in the hands of specialised laboratories using manual analytical methods.

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Introduction

Testing for vitamin D has increased exponentially in the past decade. In the United States, requests to clinical laboratories have been increasing at a rate of 80–90% per year [1]. Similarly, in Australia there was a 100-fold increase in vitamin D tests between 2000 and 2010 [2]. The demand is a consequence of the recognition of a high prevalence of deficiency in diverse populations [3] and research uncovering...
the importance of vitamin D in multiple physiological functions. The role of vitamin D in promoting absorption of dietary calcium and phosphate and increasing bone mineral density is well-recognised. However, vitamin D deficiency incurs an increased risk of conditions as disparate as insulin resistance and diabetes, cancer, autoimmune disease, cardiovascular disease and all-cause mortality [4–9].

The increase in requests for vitamin D analyses has placed pressure on clinical laboratories to offer testing procedures capable of providing results for large numbers of samples in a timely fashion. Multiple in vitro diagnostic companies have therefore been motivated to offer a vitamin D assay on their automated immunoassay platforms. Consequently, there has been an influx of new vitamin D assays onto the market and clinical laboratories can now select from a range of possible assays. However, due to the highly lipophilic nature of vitamin D, high affinity for vitamin D binding protein (DBP) as well as presence of multiple vitamin D metabolites in the circulation, vitamin D is a challenging analyte to measure accurately. These challenges are most easily met in specialist laboratories using time-consuming methods and manufacturers have had difficulty in producing high-throughput assays capable of producing results of satisfactory accuracy. In fact, a number of automated immunoassays have been withdrawn from the market because of poor analytical accuracy. Even higher-order methods, such as liquid chromatography-tandem mass spectrometry (LC-MS/MS) have had well-publicised problems with accuracy in the routine setting [10]. Furthermore, many other assays have been re-formulated as manufacturers seek to improve the performance of sub-optimal assays.

This article reviews the physiology of vitamin D metabolites and evaluates the performance of automated and chromatographic assays for 25-hydroxyvitamin D (25-OHD), the best general marker of vitamin D status. It also addresses the indications for measurement and methodologies available for measurement of the active form of vitamin D, 1,25-dihydroxyvitamin D (1,25-OH₂D).

**Physiology of vitamin D**

**Vitamin D₃ metabolism**

The primary source of vitamin D in humans is via production in the skin. This is achieved through the action of UV light on 7-dehydrocholesterol, an intermediate in the cholesterol biosynthetic pathway, also referred to as ‘provitamin D’. Cholesterol is an important component in the lipid barrier of the skin and the epidermis is an active site of cholesterol synthesis. Local synthesis thus provides a ready source of provitamin D where it is incorporated into the plasma membrane lipid bilayers of cells in the dermis and epidermis [11,12]. 7-Dehydrocholesterol is present in highest concentration in the stratum basale and stratum spinosum layers of the epidermis [13].

7-Dehydrocholesterol absorbs UVB radiation of wavelengths between 290 and 315 nm and this results in cleavage of the bond between carbon 9 and 10. The unstable 9,10-seco-sterol formed is known as ‘previtamin D₃’. Previtamin D₃ then isomerises to the more thermodynamically stable vitamin D₃ (cholecalciferol) through rotation around the bond between carbon 5 and 6 [14]. This conformational change disrupts the hydrophilic and hydrophobic interactions holding the previtamin D₃ within the cell membrane and it is ejected into the extracellular space, from where it can migrate into the circulation [15]. However, continued exposure to UV light can convert previtamin D₃ into inactive degradation products, lumisterol₃ and tachysterol₃. Similarly, exposure of the vitamin D₃ to UV radiation before it reaches the circulation will convert it into a series of inactive species (5,6-trans-vitamin D₃, suprasterol₁, suprasterol₂ [16]. These photodegradation pathways become significant with increasing UV exposure times and may provide a mechanism to avoid vitamin D toxicity from prolonged UV exposure [17].

In addition, a small amount of vitamin D₃ may also be obtained from the diet. The flesh of fatty fish (e.g. salmon, herring, tuna and mackerel) and fish liver oils are among the best sources [18]. Fortified foods, such as milk and margarine, may also contribute to vitamin D intake in some populations [19].

Vitamin D₃ requires two hydroxylation steps to obtain a biologically active form. The first of these, hydroxylation at the 25 carbon position, is performed in the liver by both microsomal and mitochondrial cytochrome P450 enzymes (including CYP2R1, CYP27A1, CYP3A4 and CYP2D5) [20,21]. The second hydroxylation step, at the 1 carbon position, is primarily performed in the proximal tubule cells of the kidney by the cytochrome P450 enzyme CYP27B. However, this enzymatic process may occur in
other tissues and has been observed in vitro in cells of the bone, placenta, adrenal gland, cerebral cortex and prostate as well as in epithelial cells of the colon, islet cells of the pancreas, keratinocytes, macrophages, T-lymphocytes and several cancer cells [21,22]. The 1,25-OH$_2$D$_3$ produced in extra-renal tissues generally does not contribute significantly to circulating levels but acts locally in an autocrine or paracrine fashion [23]. The 1$\alpha$-hydroxylase step is the primary site of regulation of 1,25-OH$_2$D homeostasis. 1,25-OH$_2$D itself, parathyroid hormone (PTH), calcium, phosphate and fibroblast growth factor 23 concentrations all exert influence on expression of the 1$\alpha$-hydroxylase enzyme [20].

**Vitamin D$_2$ metabolism**

Another vitamin D form, vitamin D$_2$ (ergocalciferol), may be obtained in small amounts from consumption of mushrooms. This form of vitamin D varies from vitamin D$_3$ in the structure of its side-chain. Vitamin D$_2$ intake becomes significant in subjects taking vitamin D$_2$-containing supplements, which are manufactured by UV irradiation of ergosterol in yeast. Vitamin D$_2$ undergoes identical hydroxylation steps for metabolic activation as vitamin D$_3$ and is acted on by the same enzymes. Vitamin D$_2$ has historically been considered functionally equivalent to vitamin D$_3$; however, vitamin D$_2$ may be less effective in raising serum total 25-OHD concentrations [24]. This may be related to variations in metabolic properties and binding affinity for DBP [25].

**Other vitamin D metabolites**

In addition to the primary pathway of vitamin D metabolism, there are also a number of minor metabolic pathways. In fact, more than 50 different vitamin D metabolites have been reported, with some exhibiting biological activity [26]. One group of compounds that has attracted recent attention are vitamin D epimers. Epimers are molecules with identical structure but different stereochemical configuration. The C3-epimers of vitamin D differ from the primary molecules only in the configuration of the hydroxyl group at the 3 carbon position, the C-3 epimer of 25-OHD$_3$ (3-epi-25-OHD$_3$) is the major form present in serum. 3-epi-25-OHD$_3$ can undergo 1$\alpha$-hydroxylation to form 3-epi-1,25-OH$_2$D$_3$, which can bind to the vitamin D receptor (VDR) and activate transcription of genes [27,28]. It appears that 3-epi-1,25-OH$_2$D$_3$ is nearly as potent as 1,25-OH$_2$D$_3$ in suppressing PTH secretion but has significantly reduced calcaemic effects [29,30].

Initial reports found C3-epimers in 23% of children under 1 year of age but none in older subjects [31]. However, 3-epi-25-OHD$_3$ has subsequently been reported to be detectable (>5 nmol/L) in 41% of samples from healthy adults. Indeed, a study using an LC-MS/MS method with a lower limit of quantification of 3-epi-25OHD$_3$ (2.5 nmol/L) was able to detect it in 99% of subjects ranging in age from neonates to over 80 years [32]. Mean concentrations of 3-epi-25-OHD$_3$ were reported as 3.8 nmol/L with a range of 2.5–59.3 nmol/L in this study [32]. Concentrations of 3-epi-25-OHD$_3$ are relatively stable over the lifespan but there is a slight decline in later life both in the absolute concentration of 3-epi-25-OHD$_3$ and proportion of total 25-OHD that is 3-epi-25-OHD$_3$ [33]. 3-epi-25-OHD$_3$ concentrations correlate with 25-OHD$_3$ concentrations in a non-linear fashion: a greater amount of 3-epimer is seen at higher 25-OHD$_3$ concentrations [33].

**Vitamin D catabolism**

The catabolism of 1,25-OH$_2$D is a multistep process which degrades vitamin D into water-soluble products, with calcitroic acid as the final product excreted in bile. The intermediates of this catabolic pathway have progressively higher water-solubility and lower or negligible activity [34]. The first step in this pathway is hydroxylation at the carbon 24 position, catalysed by a cytochrome P450 enzyme (CYP24A1) [21]. This reaction primarily occurs in the kidney; however, CYP24 mRNA has been detected in a wide range of tissues [35] and it has been speculated that it has a role in the inactivation of 1,25-OH$_2$D inside target cells [34].

While 1,25-OH$_2$D is the preferred substrate for 24-hydroxylase [35], the enzyme can also act on 25-OHD to produce 24,25-OH$_2$D, a relatively inactive metabolite. 24,25-OH$_2$D is found in the circulation at concentrations up to 24 nmol/L, with levels correlating positively with those of 25-OHD [36].
Vitamin D binding protein

DBP is the predominant serum transport protein for all vitamin D metabolites. DBP transports 95–99% of the total 25-OHD with only 1–5% carried by albumin and lipoproteins, despite their much higher concentrations in the circulation [37]. There is variability in the affinity of DBP for the various vitamin D metabolites. The highest affinity is for 25-OHD lactones, 25-OHD and 24,25-OH₂D. 1,25-OH₂D has about a 10- to 100-fold lower affinity for DBP than 25-OHD, while vitamin D itself has a still lower affinity. The vitamin D₂ metabolites bind slightly less well to human DBP their D₃ counterparts [37].

Measurement of 25-hydroxyvitamin D

The total serum 25-OHD concentration (i.e. sum of D₃ and D₂ forms) is regarded as the best single marker of vitamin D status [26]. There is essentially complete conversion of vitamin D to 25-OHD when vitamin D production and/or ingestion is below 2000 IU per day [38]. Furthermore, 25-OHD has a long half-life in the circulation, approximately 3 weeks. The circulating 25-OHD concentration also indicates the availability of substrate for local tissue production and autocrine/paracrine action of 1,25-OH₂D. In contrast, 1,25-OH₂D is tightly controlled by multiple physiological inputs and concentrations may not decrease until late into vitamin D deficiency. In addition, 1,25-OH₂D has a short half-life, approximately 4 h [39], and concentrations may be influenced by prolactin, oestradiol, testosterone, prostaglandins, bisphosphonates, corticosteroids, ketoconazole, heparin and thiazide diuretics [40].

Serum 25-OHD measurement presents a number of analytical challenges. Primary among these are its highly lipophilic nature and strong binding affinity for DBP. In addition, 25-OHD assays must be able to specifically target the molecule in the presence of a multitude of structurally-related precursor and degradation products as well as products of alternative vitamin D metabolic pathways, such as C3-epimers. Assays must also be able to detect 25-OHD₃ and 25-OHD₂ separately or, in the case of total 25-OHD assays, be able to recover them equally. The analytical methods that have been used to measure 25-OHD include competitive protein-binding assays (CPBA), immunoassays as well as chromatographic assays, which include high-performance liquid chromatography (HPLC) with UV detection and liquid chromatography-tandem mass spectrometry (LC-MS/MS).

Manual 25-hydroxyvitamin D competitive protein-binding assays and immunoassays

The first 25-OHD CPBA was published in 1971. It utilised DBP as a 25-OHD binder and a³H-25-OH₃D tracer [41]. The assay was made cumbersome by the use of organic solvent extraction and chromatography prior to assay. Simplified versions of this assay, foregoing the extraction and chromatographic steps, were automated and commercialised by the Nichols Institute. However, the Nichols assay over-estimated 25-OHD₃ and under-recovered 25-OHD₂ [42,43] and this prompted withdrawal of the assay in 2006. Roche Diagnostics has recently introduced an automated CPBA onto the market, following the withdrawal of their monoclonal vitamin D₃ immunoassay in 2010 due to poor analytical performance [44,45].

Radioimmunoassays (RIAs) for 25-OHD were first described in the mid-1980s [46,47]. The use of acetonitrile to separate 25-OHD from DBP allowed for a simplified non-chromatographic method [48]. The assay was further improved by the use of a¹²⁵I-labelled 25-OHD tracer and serum-based calibrators [49]. The assay was commercialised by DiaSorin and remains available today. The assay has been reported to have recoveries of 91–100% for 25-OHD₃ and 25-OHD₂ [50]. The goat anti-25-OHD used in the assay has a high cross-reactivity for 24,25-OH₂D, 25,26-OH₂D and 25-OH₃D-26,23-lactone; however, their concentration is proportionally small compared to the total 25-OHD concentration (<6%) [26]. The assay has a measuring range of 3.8–250 nmol/L and has been shown to have good agreement with LC-MS/MS methods in recent studies [45,51]. Therefore, laboratories not in possession of an LC-MS/MS method can use this RIA as a reference for method comparison.

Another RIA is produced commercially by IDS. This assay uses sheep polyclonal anti-25-OHD antibodies and a¹²⁵I-labelled 25-OHD tracer. This assay also cross-reacts with hydroxylated vitamin D metabolites, such as 24,25-OH₂D. The manufacturer reports a recovery of 25-OHD₂ of 75%. The assay employs a two-step procedure to release 25-OHD DBP, using sodium hydroxide and acetonitrile, and
has a measuring range of 4–400 nmol/L. Roth et al. reported a proportional bias in results compared to LC-MS/MS, with a Passing-Bablok slope of 0.64 and intercept of 9.35 nmol/L in 2008 [52]. Carter reported a positive bias compared to all-laboratory trimmed mean for the 2010/11 distribution of the UK Vitamin D External Quality Assessment Scheme (DEQAS) [53].

In addition, there are enzyme-linked immunosorbent assays (ELISAs) available commercially from IDS and Immundiagnostik. The assay from IDS uses micro-titre wells coated with sheep polyclonal anti-25-OHD antibody. Biotin-labelled 25-OHD competes for antibody binding with endogenous 25-OHD that has been dissociated from DBP in the patient sample. After incubation and washing, avidin labelled with horseradish peroxidase is added and binds to the biotin-labelled 25-OHD. The unbound avidin is then washed and tetramethylbenzidine is added, which generates a chromogenic product under the action of horseradish peroxidase. The amount of colour developed is inversely related to the concentration of 25-OHD in the sample. This assay has a 75% cross reactivity for 25-OHD2 [26]. The assay has demonstrated less than 5% bias from all-laboratory trimmed mean on the 2008–2011 DEQAS distributions [53].

The ELISA produced by Immundiagnostik is a CPBA, using micro-titre well coated with 25-OHD. Patient 25-OHD is dissociated and incubated in the well with an exogenous DBP. Anti-DBP antibody labelled with peroxidase is then added. After washing, tetramethylbenzidine is added and the colour generated is inversely related to the 25-OHD in the sample. The manufacturer reports 100% cross-reactivity is 25-OHD3, 25-OHD2 and 24,25-OH2D3.

Automated 25-hydroxyvitamin D assays

The first automated immunoassay was commercialised by DiaSorin for their LIAISON analyser. However, with increasing demand for testing there are now automated assays also available from Abbott, IDS, Roche and Siemens. Except for the current Roche assay, all current automated tests use a similar method design. Sample pre-treatment dissociates 25-OHD from DBP and it competes with exogenous 25-OHD in the assay reagent for binding sites on anti-25-OHD antibodies. Anti-25-OHD antibodies are bound to the solid-phase and the 25-OHD in the reagent is conjugated to a chemiluminescent label, or vice versa. Therefore, after washing of unbound chemiluminescent label, the light signal produced in the assay is inversely related to the 25-OHD in the patient sample. The Roche assay is a CPBA and uses exogenous DBP to capture 25-OHD in the patient sample. More details about this assay are provided below.

Abbott architect 25-OH vitamin D assay

The sample is treated with 8-anilino-1-nephtalensulfonic acid in triethanolamine methanol buffer to dissociate 25-OHD and then mixed with paramagnetic particles (the solid phase) coated with sheep polyclonal anti-vitamin D IgG. Following incubation, vitamin D conjugated with an acridinium label is added and binds to unoccupied binding sites of the anti-vitamin D IgG. The particles are captured using a magnetic field and unbound conjugate is washed. Hydrogen peroxide and sodium hydroxide are added and trigger chemiluminescence of the acridinium label.

DiaSorin LIAISON 25 OH vitamin D total assay

25-OHD is dissociated from DBP by a proprietary agent ‘ProClin 300’ in an alkaline buffer with 10% ethanol and surfactants. The assay uses goat anti-25-OHD antibodies coated onto magnetic particles. 25-OHD in the sample competes with 25-OHD conjugated with an isoluminal derivative (N-4-(-amino-butyl)-N-ethyl-isoluminol) for binding sites on the antibodies. After washing unbound conjugate, chemiluminescence is induced from the isoluminal derivative. This test is available for the LIAISON-XL as well as on the LIAISON bench top analyser.

IDS-iSYS 25-hydroxyvitamin D

The sample is treated with proprietary 25-OHD-displacing compounds in 10–20% methanol and incubated with acridinium-labelled sheep polyclonal anti-25-OHD antibodies. 25-OHD linked to
magnetic particles is then added and binds unoccupied binding sites of the anti-vitamin D antibodies. After magnetic separation and washing, the acridinium label is induced to chemiluminesce.

**Roche vitamin D total assay**

The Roche assay is a CPBA rather than an immunoassay and it uses a DBP for 25-OHD binding rather than an antibody. Pre-treatment of the sample involves a decrease in pH (with dithioreitol) followed by restoration of pH using sodium hydroxide. The sample is then incubated with ruthenium-labelled DBP. 25-OHD conjugated with biotin is then added and binds any free DBP-label. Streptavidin-coated microparticles are added and bind the 25-OHD conjugate. Microparticles are magnetically captured and, after washing, chemiluminescence is induced. The test can be run on COBAS, ELECSYS and MODULAR immunoanalysers.

**Siemens vitamin D total assay**

The sample is treated with 8-anilino-1-naphalene-sulfonic acid, ethylene glycol and diazolidinyl urea and then incubated with mouse monoclonal anti-vitamin D antibodies labelled with acridinium ester. A 25-OHD analogue conjugated to fluorescein is then added and binds with any vacant binding sites on the anti-vitamin D antibodies. Paramagnetic particles coated in monoclonal anti-fluorescein antibodies are then added and bind 25-OHD conjugate. After magnetic capture of the particles and washing, chemiluminescence is induced from the acridinium ester.

All manufacturers state that serum, either collected into plain tubes or serum separator gel tubes, is suitable for analysis. Lithium-heparin plasma is also suitable for use in all assays. EDTA-anticoagulated plasma is acceptable for all but the IDS assay; however, DiaSorin note that lithium-heparin and EDTA plasma shows a 22% bias compared with serum. 25-OHD, as well as 1,25-OH2D, has been shown to be very stable in serum and plasma and samples require only minimal attention to storage conditions [54–56].

**Precision of current automated assays**

Automated 25-OHD assays have difficulty meeting performance goals based on biological variation. The minimum performance criterion for precision is 0.75 times the intra-individual biological variation (CVI) [57]. The CVI for 25-OHD in serum is 12.1%, therefore the minimum goal for assays is 9.1% [58]. All current assays are able to meet this goal at high 25-OHD concentrations. For instance, a recent study found that at a concentration of 258 nmol/L (characterised by LC-MS/MS) the precision of the Abbott, DiaSorin, Roche and Siemens assays were 2.8–4.3% for within-run precision and 4.7–6.2% for total precision [59]. Similarly, at a concentration of 84 nmol/L the IDS assay has shown with-run precision of 1.7–4.4% and between-run precision of 3.9–5.3% [45]. However, at lower 25-OHD concentrations assays have difficulty meeting performance goals. For instance, at 24.2 nmol/L, 2 assays from 4 could meet the performance goal for total precision but at 12.8 nmol/L no assay could meet target CVs [59].

**Accuracy of current automated assays**

Current automated assays are generally able to demonstrate acceptable overall correlation with LC-MS/MS methods as a reference. For instance, recently published Passing-Bablok regression analyses for the most of the current assays have shown reasonable performance from the assays from Abbott (slopes 0.93–1.1, intercepts –1.16–5.8 nmol/L), DiaSorin (slopes 0.88–1.07, intercepts 2.8–4.6), IDS (slopes 0.83–1.2, intercepts –2.6 to 0.33) and Roche (slopes 0.83–1.2, intercepts –5.04–9.74) [45,59–62]. Assays from Abbott, DiaSorin and Roche, are able to achieve mean bias under 3% compared to LC-MS/MS and the IDS a bias under 6% [59,63]. The exception has been the assay from Siemens. Reports from the beginning of 2012 demonstrated poor performance, with Passing-Bablok regression against LC-MS/MS results giving slopes of 0.55–0.68 and intercepts 14.8–16.7 [45,60]. This poor performance was also seen in the 25-Hydroxy Vitamin D Survey offered by the College of American Pathologists [64]. More recent reports have shown somewhat improved results in comparison to LC-MS/MS on Passing-Bablok regression, with slopes 1.05–1.07 but intercepts –15.0 to –10.4 nmol/L [59,61]. However, the
assay has been shown to meet the minimum performance goal for bias based on biological variation of <15.8% [59]. Additionally, in August 2012 Siemens removed adjustments to the lower end of their master calibration curve that were contributing to the negative bias seen in some of the more recent reports.

However, a significant limitation of all current automated 25-OHD assays is the dispersion that individual results may demonstrate from the reference method result. The performance goal for total allowable error based on biological variation is <30.8% [57,58]. Bland-Altman limits of agreement for the current automated assays shows that no assay is able to report 95% of patient results within this total error limit: Abbott (−116.5 to 123.4%), DiaSorin (−57.1 to 62.9%), IDS (−50.1 to 80.0%), Roche (−69.9 to 74.0%) and Siemens (−206.7 to 230.2%) [51,59].

There are many factors that may contribute to this scatter in automated assay results compared to reference methods. One of these is the method of sample pre-treatment. Rather than use solvent extraction and chromatography clean-up steps prior to analysis, manufacturers have sought simplified sample pre-treatment to enable high sample-throughput and automation. However, this leaves the assays susceptible to interference from other lipids in the sample, particularly lipoproteins [65]. In addition, there may be interference from DBP. In lieu of solvent extraction, most current immunoassays employ a pH shift to displace 25-OHD from DBP. The validity of this approach has been recently challenged in a study of 5 automated assays compared against isotope dilution/online sold phase extraction LC-MS/MS [60]. Patient results from 4 of these assays showed a significant negative correlation with the concentration of DBP in the sample. In addition, variations in the ability to equally detect both 25-OHD3 and 25-OHD2 and in the cross-reactivity for the vitamin D metabolites and epimers of the capture antibody used, as shown in Table 1, will also contribute to discrepancies between automated assay and LC-MS/MS results.

The presence of human anti-animal antibodies (HAAA) is an additional factor that may cause erroneous immunoassay results for 25-OHD. These are more frequently a problem for immunoassays of the ‘sandwich’ design than the competitive immunoassay methods used for 25-OHD. Nevertheless, HAAA interference has been reported in the DiaSorin LIAISON assay but not their RIA method [66]. These two assays use an identical capture antibody but the RIA additionally employs a protein precipitation step prior to measurement. This prompted DiaSorin to modify the LIAISON assay. A recent report of 33 samples containing IgG and/or IgM HAAA characterised by immune dot blot by 4

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Characteristics of current automated 25-OHD assays as per manufacturers’ information.</th>
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<td>Abbott</td>
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<td>Architect</td>
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<td></td>
<td>Sheep polyclonal Acridinium</td>
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<tr>
<td>Sample volume (μL)</td>
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\(a\) Intermediate precision.

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automated assays has shown the DiaSorin, Roche and Siemens assays to give mean results within 5% of an LC-MS/MS reference method, while the Abbott assay demonstrated a mild (24%) mean over-recovery of these samples [59].

Despite the numerous factors contributing to variability in results, automated 25-OHD assays have generally been improving. Results submitted to UK Vitamin D External Quality Assessment Scheme have demonstrated a gradual reduction in inter-laboratory coefficients of variation from 30% in 1995 to 15% in 2011 [67]. As manufacturers continue to confront the issues raised by the use of automated high-throughput assays, further improvements are expected.

Chromatographic 25-hydroxyvitamin D assays

The other main group of 25-OHD assays is those using chromatography. The two predominant method types of this group are HPLC with UV detection and LC-MS/MS. Chromatographic methods have the advantage of being able to separately measure 25-OHД3 and 25-OHД2 as well as other metabolites, such as 24,25-OHД3. However, these assays are limited by lower sample throughput capabilities and requirement for specialist staff.

High-performance liquid chromatography assays

HPLC methods for 25-OHD measurement were developed in the 1970s. The first assays used normal phase separation on silica columns [68,69], while reverse-phase chromatography, generally on C18 columns, became popular in later methods [70]. Following chromatography, vitamin D metabolites are most commonly quantitated by measuring absorption of UV light at 265 nm, although electrochemical detection methods are also used. Laboratories may develop in-house HPLC methods for 25OHD; however, HPLC ‘kit’ applications from Chromsystems, ESA, Immunodiagnostik and others are also available for purchase. HPLC methods are capable of resolving 25-OHД3 and 25-OHД2 as well as the parent compounds and 1,25-OHД3 and 24,25-OHД3 metabolites [71].

HPLC methods have the capacity to be very accurate, particularly in the hands of experienced personnel. In less experienced hands, there is the potential to misinterpret spurious UV-absorbing peaks as 25-OHД2 and therefore over-estimate 25-OHD results [72]. Lipids in patient samples may disturb the elution of 25-OHD metabolites. Therefore, clean-up steps, such as liquid–liquid and liquid–solid extraction, are necessary for reliable results [42,71].

Modern HPLC methods show acceptable performance characteristics. Between-run precision has been reported as 2.6–4.9% for 25-OHД3 and 3.2–13% for 25-OHД2. There is also good correlation with LC-MS/MS (r > 0.995) for both 25-OHД3 and 25-OHД2 and linear regression gives equations of \( y = 1.01x - 12.05 \) nmol/L for 25-OHД3 and \( y = 0.902x - 1.415 \) nmol/L for 25-OHД2 [71]. The main disadvantage of HPLC methods are a large sample volume requirements, slow sample throughput, need for specialist staff and high equipment costs [72].

Liquid chromatography-tandem mass spectrometry

LC-MS/MS is widely regarded as the best available technique for 25-OHD quantification. The increased analytical specificity provided by the tandem mass spectrometry detection offers the potential for high accuracy and overcomes many of the difficulties associated with immunoassays. Indeed, recognised reference methods for 25-OHД3 and 25-OHД2 have been developed utilising isotope dilution LC-MS/MS methodology [73,74]. LC-MS/MS methods are also in routine use and represent approximately 11% of DEQAS participants [67].

The precision of LC-MS/MS assays has been shown to be excellent. For a method using robotic liquid-handling technology, a within-run CV of 2.4% and between-run CV of 2.0% have been reported [45]. Other LC-MS/MS methods have reported within-run CVs of 4.1–7.1% and between-run CVs of 6.1–12.1% [45,75].

LC-MS/MS methods are not immune from interferences. One category of interferences specific to mass spectrometry methods is isobars. These are compounds with the same molecular weight as 25-OHD that form precursor and product ion pairs with the same mass-to-charge ratios as those from 25-
OHĐ. Isobars are a particular issue with routine LC-MS/MS assays that typically use short chromatographic run times, where isobars may co-elute with 25-OHĐ analytes or internal standards. 7α-hydroxy-4-cholesten-3-one is an isobar of particular relevance. It is a bile acid precursor found in the serum of 4 out of 5 healthy volunteers [75]. Another isobar that may be affect results for particular patients is 1α-hydroxyvitamin-D$_3$, a therapeutic agent used in the management of secondary hyperparathyroidism in chronic kidney disease. Shah et al. found that in 5 healthy volunteers isobars could contribute up to 38.7% of the total 25-OHĐ concentration [75].

Most routine LC-MS/MS methods do not distinguish 3-epi-25-OHĐ$_3$ from 25-OHĐ$_3$. Therefore, the 3-epi-25-OHĐ$_3$ present in the patient sample will contribute to the total 25-OHĐ reported by the assay. This has been found to cause in misclassification of vitamin D deficiency in 9% of patients under 1 year of age and 3% of patients aged 1–80 years [33]. Routine LC-MS/MS methods do exhibit a persistent positive bias on DEQAS [67] and isobar and 3-epi-25-OHĐ$_3$ interference may contribute to this finding. LC-MS/MS methods with the ability to identify these interferences have been described [32,33,75]. However, the chromatographic separation of epimers and isobars requires longer run times, reducing the practicality of implementation into the routine setting.

LC-MS/MS assays have previously been shown poor agreement to one another. An inter-laboratory coefficient of variation of 16.4%, which is commensurate with immunoassays, has been reported [76]. One of the factors contributing to this may have been the lack of reference materials or a reference measurement procedure. This was addressed by the introduction of the NIST standard reference materials, SRM972 and SRM2972, in 2009. The agreement between different LS-MS/MS assays has been shown to improve when a common standard is used [76]. Therefore, it is expected that the introduction of these standard reference materials will improve inter-laboratory agreement of LC-MS/MS methods. Certainly, a report of two different LC-MS/MS methods in the era of SRM972 has shown very good agreement between assays despite difference in sample preparation and extraction procedures: mean bias 1.4 nmol/L with Bland-Altman 95% limits of agreement of –3.5–6.3 nmol/L [45].

A major disadvantage of routine use of LC-MS/MS 25-OHĐ analysis is limited sample throughput. This challenge has been addressed in a number of ways. One approach is to have multiple liquid chromatography systems attached to each tandem mass spectrometer. Such ‘multiplexed’ systems are available commercially (e.g. Thermo Scientific TLX-4 System, Applied Biosystems Cliquid MPX-2). In these systems, samples are introduced into the chromatography columns in a staggered manner and are directed into the mass spectrometer only at their peak time of elution. A novel approach described by Netzel et al. is the use of a number of different, but closely related, derivatising reagents for different samples [77]. This group has used triazoline-diones with 5 functional groups of different mass attached for derivatisation. As the mass spectrometer can distinguish the 25-OHĐ3 and 25-OHĐ2 derivatised with different reagents, samples from 5 patients can be introduced into the LC-MS/MS in a single injection. When this is combined with multiplexed LC-MS/MS systems, a throughput of 300 specimens per hour is achievable.

### 1,25-Dihydroxyvitamin D measurement

**Indications for measurement**

Although 25-OHĐ is generally the preferred marker of vitamin D status, 1,25-OH$_2$D measurement is important in a limited number of circumstances. In particular, the 1,25-OH$_2$D concentration is clinically relevant in circumstances in which there may be a disorder of 1α-hydroxylation. This most commonly occurs in the context of chronic kidney disease (CKD). A number of mechanisms may contribute to impaired 1α-hydroxylation in CKD: decreased quantity of 1α-hydroxylase due to the decreased kidney mass, decreased delivery of 25-OHĐ to the enzyme in the proximal tubular cells due to the decrease in glomerular filtration rate as well as down-regulation of 1α-hydroxylase expression caused by increased phosphate and fibroblast growth factor 23 concentration [78,79]. A less common disorder of 1α-hydroxylase activity is vitamin D-dependent rickets type 1. Measurement of 1,25-OH$_2$D is also useful in the diagnosis of hypophosphataemic rickets and vitamin D dependent rickets type 2. In hypophosphataemic rickets, 1,25-OH$_2$D concentrations are low or at the lower end of the reference interval, while in vitamin D dependent rickets there is an inherited disorder of the vitamin D receptor (VDR) and very high serum 1,25-OH$_2$D concentrations [80].
Measurement of 1,25-OH₂D may also be indicated in some cases of hypercalcaemia. Over-
expression of 1α-hydroxylase can produce a non-PTH-mediated hypercalcaemia in which the 25-
OHD concentration is normal but the 1,25-OH₂D level is raised. This scenario may occur in patients
with malignant lymphoma, sarcoidosis and other conditions characterised by granuloma formation
(e.g. tuberculosis and Wegener’s granulomatosis) as well as ingestion of excess calcitriol [81].

Measurement of 1,25-dihydroxyvitamin D

The accurate measurement of 1,25-OH₂D is particularly challenging. 1,25-OH₂D is highly lipophilic
and circulates at picomolar concentrations. Furthermore, it must be distinguished from 25-OHD, which
is structurally similar and circulating at 1000-fold higher concentrations. Therefore, a high degree of
both analytical sensitivity and specificity is required for successful 1,25-OH₂D assays.

The first assay to measure 1,25-OH₂D was a CPBA which used VDR from the intestines of chickens
and a 3H-labelled 1,25-OH₂D tracer [82]. The assay required cumbersome extraction and purification
procedures and it was necessary for chickens to be sacrificed. Subsequently, calf thymus was identified
as a good source of VDR and this formed the basis for an assay using solid-phase extraction and silica
cartridge purification [83]. A significant development was the production of a radioimmunoassay using
polyclonal sheep anti-1,25-OH₂D3 antibodies and an 125I-labelled tracer. This assay did not require
sample pre-purification by HPLC, used serum-based standards and did not require internal standards
to determine individual sample recovery [84].

Most clinical laboratories currently offering 1,25-OH₂D assays use kit methods marketed
commercially [85]. The two most commonly used methods are from DiaSorin and IDS. Both assays use a
competitive RIA design with 125I as the radio-label but differ in sample pre-treatment procedures. The
DiaSorin method uses solid-phase extraction and silica purification using organic solvents, while the
IDS method using solid-phase immunoextraction. Both assays detect 1,25-OH₂D₂; the DiaSorin assay
shows 100% recovery, while the IDS assay has a 91% recovery for 1,2-5OH₂D₂ [86,87].

1,25-OH₂D enzyme immunoassays are commercially marketed by IDS and Immundiagnostik. The
IDS assay uses solid-phase immunoextraction and colorimetric detection and has been shown to have
good correlation with a calf-thymus radio-receptor assay and acceptable performance on DEQAS
proficiency survey, but may underestimate 1,25-OH₂D₂ [86]. Less has been published on the perfor-
ance of the Immundiagnostik assay. A limitation of 1,25-OH₂D immunoassays generally is that a 1,25-
OH₂D antibodies produced commercially exhibit a degree of cross-reactivity with 25-OHD and 24,25-
OH₂D, which will limit their specificity [88].

LC-MS/MS methods have the advantage of very high levels of analytical specificity and a number
of LC-MS/MS methods have also been developed for 1,25-OH₂D₂ measurement. A challenge for LC-
MS/MS methods is that 1,25-OH₂D ions poorly with both atmospheric pressure chemical ionisa-
tion and electrospray ionisation. This is generally overcome by the use of derivatisation; however,
derivatisation can be avoided by methods that use large sample volumes (2 mL) [89]. An advantage of
LC-MS/MS methods is the ability to simultaneously measure 1,25-OH₂D₃, 1,25-OH₂D₂, 24,25-OH₂D, 25-OHD₃ and 25-OHD₂. The initial methods used to measure multiple metabolites had poor lower
limits of quantification but improvements in techniques have decreased limit of quantification to 12 pmol/L [90,91]. The use of immunoextraction with monoclonal anti-1,25-OH₂D antibodies prior to
derivatisation and LC-MS/MS measurement allows LC-MS/MS assays to achieve low limits of quan-
tification (3 pmol/L for 1,25-OH₂D₃ and 1.5 pmol/L for 1,25-OH₂D₂) with an instrument cycle time
under 5 min [88].

Conclusions

The analysis of vitamin D and its metabolites is a rapidly evolving field. The recognition of the
importance of vitamin D beyond simply skeletal health and a high prevalence of deficiency in diverse
populations has created a rapid increase in demand for vitamin D testing. Consequently, vitamin D
analysis has moved from specialist laboratories using highly manual assays to routine laboratories
using automated assays on high-throughput analysers. The difficulty of providing accurate automated
25-OHD assays has been highlighted by the withdrawal of a number of commercial assays from the
market due to analytical problems. Most automated assays currently available show good overall agreement with reference methods. The ongoing issue with current assays is the degree of dispersion that individual patient results may show from the true result. This scatter in results appears to be related to interference from endogenous lipids and DBP in patient samples, variations in cross-reactivity to 3-epi-25-OHD3 and other vitamin D metabolites as well as susceptibility to HAAA inference. Chromatographic methods, particularly LC-MS/MS, provide an alternative for 25-OHD analysis but are not immune from interferences of their own and have limited sample throughput. Assessment of 1,25-OH2D may also be appropriate in some circumstances. However, due to the technical difficulties associated with the measurement of this analyte, 1,25-OH2D measurement largely remains the domain of specialist laboratories. Additionally, as further understanding of other vitamin D metabolites is reached and mass spectrometry techniques improve, the measurement of metabolites such as 24,25-OH2D may begin to have a role in routine practice.

Practice points

- Vitamin D status is primarily assessed using automated laboratory assays for the measurement of total 25-hydroxyvitamin D in serum (i.e. sum of D3 and D2 forms).
- Automated 25-hydroxyvitamin D assays show good precision at high concentrations but have difficulties to meet analytical goals at low concentrations.
- Automated 25-hydroxyvitamin D assays generally show good overall correlation with reference methods but discrepancies persist for individual patient samples.
- 1,25-Dihydroxyvitamin D measurement has clinical utility in a small number of situations.

Research agenda

- Ongoing research is required into the precision and accuracy of automated 25-hydroxyvitamin D assays as new assays continue to be introduced and established assays re-formulated.
- The improvement of the accuracy of automated 25-hydroxyvitamin D assays for all patient samples is a priority.
- The clinical and analytical role of vitamin D metabolites other than 25-hydroxyvitamin D and 1,25 dihydroxyvitamin D (e.g. C3-epimers) requires further investigation
- Improvements to the sample-throughput capacities of liquid chromatography-mass spectrometry methods for 25-hydroxyvitamin D would enable more widespread implementation of this technology.

Summary

There has been a dramatic worldwide increase in demand for the laboratory assessment of patients’ vitamin D status. Although laboratories are able to measure a number of vitamin D metabolites, it is total serum 25-hydroxyvitamin D (i.e. sum of D3 and D2 forms) which is considered the best marker of status in most patients. To cope with the increased demand for testing laboratories have increasingly be using automated 25-OHD assays and a number of new automated assays have been recently introduced into routine use. The measurement of 25-OHD is technically difficult due to its highly lipophilic nature, tight binding to vitamin D binding protein, the presence of multiple structurally related compounds in the circulation as well as the requirement to equally measure the D3 and D2 forms of the vitamin. These difficulties challenge the precision of current automated 25-OHD assays, which show the ability to meet performance goals at high concentrations of the analyte but fail to meet goals at lower concentrations. Overall, these assays generally show good agreement with reference
methods but individual patient samples may demonstrate significant deviation from the results of reference methods. Chromatographic methods for 25-OHD, such as HPLC and LC-MS/MS, provide the potential for greater analytical accuracy but remain hampered by limited sample throughput. 1,25-OH2D is the active form of the vitamin, which is important to measure in clinical situations where there may be a disorder of 1α-hydroxylase activity. 1,25-OH2D presents even greater technical challenges to the analyst, largely due to it circulating at concentrations 1000-fold lower than 25-hydroxyvitamin D. Assays for 1,25-OH2D have therefore remained in the hands of specialised laboratories using manual methods.

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