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1

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.

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

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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-OH_2D).

Physiology of vitamin D

Vitamin D_3 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 UV_B 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 D_3'. Previtamin D_3 then isomerises to the more thermodynamically stable vitamin D_3 (cholecalciferol) through rotation around the bond between carbon 5 and 6 [14]. This conformational change disrupts the hydrophilic and hydrophobic interactions holding the previtamin D_3 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 D_3 into inactive degradation products, lumisterol$_3$ and tachysterol$_3$. Similarly, exposure of the vitamin D_3 to UV radiation before it reaches the circulation will convert it into a series of inactive species (5,6-trans-vitamin D_3, suprasterol$_1$, suprasterol$_2$ [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 D_3 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 D_3 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

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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_2D_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 1α-hydroxylase step is the primary site of regulation of 1,25-OH_2D homeostasis. 1,25-OH_2D itself, parathyroid hormone (PTH), calcium, phosphate and fibroblast growth factor 23 concentrations all exert influence on expression of the 1α-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 1α-hydroxylation to form 3-epi-1,25-OH_2D_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_2D_3 is nearly as potent as 1,25-OH_2D_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-25OHD3 (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_2D 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_2D inside target cells [34].

While 1,25-OH_2D is the preferred substrate for 24-hydroxylase [35], the enzyme can also act on 25-OHD to produce 24,25-OH_2D, a relatively inactive metabolite. 24,25-OH_2D is found in the circulation at concentrations up to 24 nmol/L, with levels correlating positively with those of 25-OHD [36].

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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-OH_2D. 1,25-OH_2D 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 D_2 metabolites bind slightly less well to human DBP their D_3 counterparts [37].

Measurement of 25-hydroxyvitamin D

The total serum 25-OHD concentration (i.e. sum of D_3 and D_2 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-OH_2D. In contrast, 1,25-OH_2D is tightly controlled by multiple physiological inputs and concentrations may not decrease until late into vitamin D deficiency. In addition, 1,25-OH_2D 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-OHD_3 and 25-OHD_2 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 a ^{3}H-25-OHD_3 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 overestimated 25-OHD_3 and under-recovered 25-OHD_2 [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 D_3 immunoassay in 2010 due to poor analytical performance [44,45].

Radioimmunoassays (RIAs) for 25-OHD_3 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 a ^{125}I-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-OHD_3 and 25-OHD_2 [50]. The goat anti-25-OHD used in the assay has a high cross-reactivity for 24,25-OH_2D, 25,26-OH_2D and 25-OHD_3-26,23-lactone; however, their concentration is proportionally small compared to the total 25-OHD concentration ($\leq$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 a ^{125}I-labelled 25-OHD tracer. This assay also cross-reacts with hydroxylated vitamin D metabolites, such as 24,25-OH_2D. The manufacturer reports a recovery of 25-OHD_2 of 75%. The assay employs a two-step procedure to release 25-OHD DBP, using sodium hydroxide and acetonitrile, and

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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-OHD_2 [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-OHD_3, 25-OHD_2 and 24,25-OH_2D_3.

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

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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 biding 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-OH_2D, 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 (CV_I) [57]. The CV_I 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. At 24.2 nmol/L 2 out of 4 assays 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 0.83–1.21, 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-Hydoxy 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

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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]. It 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-OHD_3 and 25-OHD_2 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 1
Characteristics of current automated 25-OHD assays as per manufacturers' information.

	Abbott	DiaSorin	IDS	Roche	Siemens
Instrument	Architect	Liaison	iSYS	Elecsys/Modular/ Cobas	Centaur/Centaur XP
Antibody	Sheep polyclonal	Goat polyclonal	Sheep polyclonal	DBP	Mouse monoclonal
Label	Acridinium	Isoluminol	Acridinium ester	Ruthenium	Acridinium ester
Sample volume (μL)	60	25	10	15	20
LoB	4.8	Not stated	4.5	5.0	4.0
LoD	7.8	Not stated	9.0	7.5	8.0
LoQ	20	10.0	13.8	12.5	8.8
Reportable range	20–400	10–375	12.5–350	12.5–150	9.3–375
Within-run CV (%)	≤3.7	≤7.7	≤12.1	≤7.2	≤7.0
Total CV (%)	<4.6	≤12.6	<16.9	≤12.6[a]	≤11.1
Recovery of Vitamin D Metabolites (%)					
25-OHD_3	105	100	100	100	101
25-OHD_2	82	100	100	92	105
Vitamin D_3	0.1	1.9	2.7	'Not detectable'	0.3
Vitamin D_2	0.2	1.9	2.7	'Not detectable'	0.5
1,25-OH_2D_3	Not stated	9.3	Not stated	'Not detectable'	1.0
1,25-OH_2D_2	12.6	6.7	Not stated	'Not detectable'	4.0
3-epi-25-OHD_3	2.7	1.3	Not stated	91	1.1
24,25-OH_2D_3	112	Not stated	≥100	149	Not stated

[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-OHD_3 and 25-OHD_2 as well as other metabolites, such as 24,25-OH_2D_3. 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-OHD_3 and 25-OHD_2 as well as the parent compounds and 1,25-OH_2D and 24,25-OH_2D 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-OHD_2 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-OHD_3 and 3.2–13% for 25-OHD_2. There is also good correlation with LC-MS/MS ($r > 0.995$) for both 25-OHD_3 and 25-OHD_2 and linear regression gives equations of $y = 1.01x - 12.05$ nmol/L for 25-OHD_3 and $y = 0.902x - 1.415$ nmol/L for 25-OHD_2 [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-OHD_3 and 25-OHD_2 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-

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OHD. 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-OHD 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-OHD concentration [75].

Most routine LC-MS/MS methods do not distinguish 3-epi-25-OHD_3 from 25-OHD_3. Therefore, the 3-epi-25-OHD_3 present in the patient sample will contribute to the total 25-OHD 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-OHD_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-OHD 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-OHD_3 and 25-OHD_2 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-OHD is generally the preferred marker of vitamin D status, 1,25-OH_2D measurement is important in a limited number of circumstances. In particular, the 1,25-OH_2D 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-OHD 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_2D is also useful in the diagnosis of hypophosphataemic rickets and vitamin D dependent rickets type 2. In hypophosphataemic rickets, 1,25-OH_2D 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_2D concentrations [80].

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Measurement of 1,25-OH_2D 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-OH_2D 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-OH_2D is particularly challenging. 1,25-OH_2D 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-OH_2D assays.

The first assay to measure 1,25-OH_2D was a CPBA which used VDR from the intestines of chickens and a ^{3}H-labelled 1,25-OH_2D 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 sold-phase extraction and silica cartridge purification [83]. A significant development was the production of a radioimmunoassay using polyclonal sheep anti-1,25-OH_2D_3 antibodies and an ^{125}I-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-OH_2D 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 ^{125}I 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-OH_2D_2: the DiaSorin assay shows 100% recovery, while the IDS assay has a 91% recovery for 1,2-5OH_2D_2 [86,87].

1,25-OH_2D 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-OH_2D_2 [86]. Less has been published on the performance of the Immundiagnostik assay. A limitation of 1,25-OH_2D immunoassays generally is that a 1,25-OH_2D antibodies produced commercially exhibit a degree of cross-reactivity with 25-OHD and 24,2-5OH_2D, 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-OH_2D_2 measurement. A challenge for LC-MS/MS methods is that 1,25-OH_2D ionises poorly with both atmospheric pressure chemical ionisation 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-OH_2D_3, 1,25-OH_2D_2, 24,25-OH_2D, 25-OHD_3 and 25-OHD_2. 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-OH_2D antibodies prior to derivatisation and LC-MS/MS measurement allows LC-MS/MS assays to achieve low limits of quantification (3 pmol/L for 1,25-OH_2D_3 and 1.5 pmol/L for 1,25-OH_2D_2) 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

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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-OHD_3 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-OH_2D may also be appropriate in some circumstances. However, due to the technical difficulties associated with the measurement of this analyte, 1,25-OH_2D 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-OH_2D 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 D_3 and D_2 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 D_3 and D_2 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 D_3 and D_2 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

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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-OH_2D 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-OH_2D 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-OH_2D have therefore remained in the hands of specialised laboratories using manual methods.

References

[1] Singh R. Are clinical laboratories prepared for accurate testing of 25-hydroxyvitamin D? Clinical Chemistry 2008;54:221–331.
[2] Medical Benefits Reviews Task Group. Vitamin D testing. Review of the funding arrangements for pathology services. Australian Government, Department of Health and Ageing; 2011. p. 60–1.
[3] van Schoor NM, Lips P. Worldwide vitamin D status. Best Practice & Research Clinical Endocrinology & Metabolism 2011; 25:671–80.
[4] von Hurst PR, Stonehouse W, Coad J. Vitamin D supplementation reduces insulin resistance in South Asian women living in New Zealand who are insulin resistant and vitamin D deficient - a randomised, placebo-controlled trial. British Journal of Nutrition 2010;103:549–55.
[5] Hyppönen E, Läärä E, Reunanen A, et al. Intake of vitamin D and risk of type 1 diabetes: a birth-cohort study. Lancet 2001; 358:1500–3.
[6] Lappe JM, Travers-Gustafson D, Davies KM, et al. Vitamin D and calcium supplementation reduces cancer risk: results of a randomized trial. American Journal of Clinical Nutrition 2007;85:1581–91.
[7] Souberbielle JC, Body JJ, Lappe JM, et al. Vitamin D and musculoskeletal health, cardiovascular disease, autoimmunity and cancer: recommendations for clinical practice. Autoimmunity Reviews 2010;9:709–15.
[8] Wang TJ, Pencina MJ, Booth SL, et al. Vitamin D deficiency and risk of cardiovascular disease. Circulation 2008;117:503–11.
[9] Autier P, Gandini S. Vitamin D supplementation and total mortality: a meta-analysis of randomized controlled trials. Archives of Internal Medicine 2007;167:1730–7.
[10] Pollack A. Quest acknowledges errors in vitamin D tests. New York Times. Available at: http://www.nytimes.com/2009/01/08/business/08labtest.html?_r=0 [accessed 16.02.12].
[11] Glossmann HH. Origin of 7-dehydrocholesterol (provitamin D) in the skin. Journal of Investigative Dermatology 2010; 130:2139–41.
[12] Tsiaras WG, Weinstock MA. Factors influencing vitamin D status. Acta Dermato- Venereologica 2011;91:115–24.
[13] Holick MF, MacLaughlin JA, Clark MB, et al. Photosynthesis of previtamin D3 in human skin and the physiologic consequences. Science 1980;210:203–5.
*[14] Chen TC, Lu Z, Holick MF. Photobiology of vitamin D. In: Hollick MF, editor. Vitamin D: physiology, molecular biology, and clinical applications. New York: Humana Press; 2010. p. 35–60.
[15] Tian XQ, Chen TC, Lu Z, et al. Characterization of the translocation process of vitamin D3 from the skin into the circulation. Endocrinology 1994;132:655–61.
[16] Webb AR, DeCosta BR, Holick MF. Sunlight regulates the cutaneous production of vitamin D3 by causing its photodegradation. Journal of Clinical Endocrinology and Metabolism 1989;68:882–7.
[17] Holick MF, MacLaughlin JA, Doppelt SH. Regulation of cutaneous previtamin D3 photosynthesis in man: skin pigment is not an essential regulator. Science 1981;211:590–3.
[18] Institute of Medicine. Food and nutrition board. Dietary reference intakes for calcium and vitamin D. Washington, DC: National Academy Press; 2010.
[19] National Institutes of Health, Office of Dietary Supplements. Dietary supplement fact sheet: vitamin D. Available at: http://ods.od.nih.gov/factsheets/VitaminD-HealthProfessional/ [accessed 28.01.13].
*[20] Christakos S, Ajibade DV, Dhawan P, et al. Vitamin D: metabolism. Endocrinology and Metabolism Clinics of North America 2010;39:243–53.
[21] Lehmann B. The vitamin D3 pathway in human skin and its role for regulation of biological processes. Photochemisstry and Photobiology 2005;81:1246–51.
[22] Zehnder D, Bland R, Williams MC, et al. Extrarenal expression of 25-hydroyxvitamin D3-1alpha-hydroxylase. Journal of Clinical Endocrinology and Metabolism 2001;86:888–94.
[23] Prentice A, Goldberg GR, Schoenmakers I. Vitamin D across the lifecycle: physiology and biomarkers. American Journal of Clinical Nutrition 2008;88(suppl.):500S–6S.
[24] Tripkovic L, Lambert H, Hart K, et al. Comparison of vitamin D2 and vitamin D3 supplementation in raising serum 25-hydroxyvitamin D status: a systematic review and meta-analysis. American Journal of Clinical Nutrition 2012;95: 1357–64.
[25] Houghton LA, Vieth R. The case against ergocalciferol (vitamin D2) as a vitamin supplement. American Journal of Clinical Nutrition 2006;84:694–7.
[26] Zerwekh JE. Blood biomarkers of vitamin D status. American Journal of Clinical Nutrition 2008;87(suppl.):1087S–91S.
[27] Rehan VK, Torday JS, Peleg S, et al. 1a,25-dihydroxy-3-epi-vitamin D, a natural metabolite of 1a,25-dihydroxy vitamin D3: production and biological activity studies in pulmonary alveolar type II cells. Molecular Genetics and Metabolism 2002; 76:46–56.
[28] Messerlian S, Gao X, St. Arnaud R. The 3-epi and 24-oxo-derivatives of 1a,25-dihydroxyvitamin D3 stimulate transcription through the vitamin D receptor. Journal of Steroid Biochemistry and Molecular Biology 2000;72:29–34.

Please cite this article in press as: Farrell C-J, Herrmann M, Determination of vitamin D and its metabolites, Best Practice & Research Clinical Endocrinology & Metabolism (2013), http://dx.doi.org/10.1016/j.beem.2013.06.001

[29] Brown AJ, Ritter C, Slatopolsky E, et al. 1a,25-Dihydroxy-3-epi-vitamin D3, a natural metabolite of 1a-,25-dihydroxyvitamin D3, is a potent suppressor of parathyroid hormone secretion. Journal of Cellular Biochemistry 1999; 73:106–13.
[30] Fleet JC, Bradley J, Reddy GS, et al. 1a,25-(OH)2-Vitamin D3 analogs with minimal in vivo calcemic activity can stimulate significant transepithelium calcium transport and mRNA expression in vitro. Archives of Biochemistry and Biophysics 1996;329:228–34.
[31] Singh RJ, Taylor RL, Reddy GS, Grebe SKG. C-3 Epimers can account for a significant proportion of total circulating 25-hdyroxyvitamin D in infants, complicating accurate measurement and interpretation of vitamin D status. Journal of Clinical Endocrinology and Metabolism 2006;91:2055–61.
*[32] Lensmeyer G, Poquette M, Wiebe D, Binkley N. The C-3 epimer of 25-hydroxyvitamin D3 is present in adult serum. Journal of Clinical Endocrinology and Metabolism 2012;97:163–8.
[33] Strathmann FG, Sadilkova K, Laha TJ, et al. 3-epi-25 hydroxyvitamin D concentrations are not correlated with age in a cohort of infants and adults. Clinica Chimica Acta 2012;413:203–6.
[34] Jones G, Strugnell SA, DeLuca HF. Current understanding of the molecullar actions of vitamin D. Physiological Reviews 1998;78:1195–231.
[35] Shinki T, Jin CH, Nishimura A, et al. Parathyroid hormone inhibits 25-hydroxyvitmain D3-24-hydroxylase mRNA expression stimulation of 1 alpha25-dihydroxyvitamin D3 in rat kidney but not in intestine. Journal of Biological Chemistry 1992;267:13757–62.
[36] Bosworth CR, Levin G, Robinson-Cohen C, et al. The serum 24,25-dihydroxyvitamin D concentration, a marker of vitamin D catabolism, is reduced in chronic kidney disease. Kidney International 2012;82:693–700.
[37] Bouillon R. The vitamin D binding protein. In: Feldman D, Pike JW, Adams JS, editors. Vitamin D. 3rd ed. London: Academic Press; 2011. p. 57–72.
[38] Heaney RP, Armas LAG, Shary JR, et al. 25-Hydroxylation of vitamin D3: relation to circulating vitamin D3 under various input conditions. Amerian Journal of Clinical Nutrition 2008;87:1738–42.
*[39] Holick MF, Binkley NC, Bischoff-Ferrari HA, et al. Evaluation, treatment, and prevention of vitamin D deficiency: an Endocrine Society Clinical Practice Guideline. Journal of Clinical Endocrinology and Metabolism 2011;96:1911–30.
[40] Epstein S, Schnider AE. Drug and hormone effects on vitamin D metabolism. In: Feldman D, Pike JW& Glorieux FH, editors. Vitamin. 2nd ed. Amsterdam: Elsevier; 2005. p. 1253–91.
[41] Haddad JG, Chyu KJ. Competitive protein-binding radioassay for 25-hydroxycholecalciferol. Journal of Clinical Endocrinology and Metabolism 1971;33:992–5.
[42] Lensmeyer GL, Wiebe DA, Binkley N, Drezner MK. HPLC method for 25-hydroxyvitamin D measurement: comparison with comtemporary assays. Clinical Chemistry 2006;52:1120–6.
*[43] Carter GD, Carter R, Jones J, Berry J. How accurate are assays for 25-hydroxyvitamin D? Data from the internation vitamin D external quality assessment Scheme. Clinical Chemistry 2004;50:2195–7.
[44] Connell AB, Jenkins N, Black M, et al. Overreporting of vitamin D deficiency with the Roche Elecsys vitamin D3 (25-OH) method. Pathology 2011;43:368–71.
[45] Farrell CJ, Martin S, McWhinney B, et al. State-of-the-art vitamin D assays: a comparison of automated immunoassays with liquid chromatography-tandem mass spectrometry methods. Clinical Chemistry 2012;58:531–42.
[46] Hummer L, Nilas L, Tjelleeen L, Christiansen C. A selective and simplified radioassay of 25-hydroxyvitamin D3. Scandinavian Journal of Clinical and Laboratory Investigation 1984;44:163–7.
[47] Bouillon R, Van Herck E, Jan I, et al. Two direct (nonchromatographic) assays for 25-hydroxyvitamin D. Clinical Chemistry 1984;30:1731–6.
[48] Hollis BW, Napoli JL. Improved radioimmunoassay for vitamin D and its use in assessing vitamin D status. Clinical Chemistry 1985;31:1815–9.
[49] Hollis BW, Kamerud JQ, Selvaag SR, et al. Determination of vitamin D status by radioimmunoassay with an 125I-labeled tracer. Clinical Chemistry 1993;39:529–33.
[50] Hollis BW. Comparison of commercially available (125)I-based RIA methods for the determination of circulating 25-hydroxyvitamin D. Clinical Chemistry 2000;46:1657–61.
[51] Janssen MJW, Wielders JPM, Bekker CC, et al. Multicenter comparison study of current methods to measure 25-hydroxyvitamin D in serum. Nederlands Tijdschrift voor Klinische Chemie en Laboratoriumgeneeskende 2012;27:223–6.
[52] Roth HJ, Schimdt-Gayk H, Weber H, Niederau C. Accuracy and clinical implications of seven 25-hydroxyvitamin D methods compared with liquid chromatography-tandem mass spectrometry as a reference. Annals of Clinical Biochemistry 2008;25:153–9.
[53] Carter GD. Are 25-hydroxyvitamin D assays fit for purpose? In: Renz H, Tauber R, editors. Advances in clinical chemistry and laboratory medicine. Berlin: Walter de Gruyter; 2012. p. 58–60.
*[54] Hollis B. Measuring 25-hydroxyvitamin D in a clinical environment: challenges and needs. American Journal of Clinical Nutrition 2008;88(suppl.):507S–10S.
[55] Wielders JPM, Wijnberg FA. Preanalytical stability of 25(OH)-vitamin D3 in human blood or serum at room temperature: solid as a rock. Clinical Chemistry 2009;55:1584–95.
[56] Antoniucci DM, Black DM, Sellmeyer DE. Serum 25-hydroxyvitamin D is unaffected by multiple freeze-thaw cycles. Clinical Chemistry 2005;51:258–61.
[57] Fraser C. Biological variation from principles to practice. Washington, DC: AACC Press; 2001.
[58] Viljoen A, Singh DK, Farrington K, Twomey PJ. Analytical quality goals for 25-vitamin D based on biological variation. Journal of Clinical Laboratry Analysis 2011;25:130–3.
[59] Farrell C, Soldo J, Williams P, Herrmann M. 25-hydroxyvitamin D testing: challenging the performance of current automated immunoassays. Clinical Chemistry and Laboratory Medicine 2012;50:1953–63.
*[60] Heijboer AC, Blankenstein RA, Kema IP, Buijs M. Accuracy of six routine 25-hydroxyvitamin D assays: influence of vitamin D binding protein concentration. Clinical Chemistry 2012;58:543–8.
[61] Chen Y, Kinney L, Bozovic A, et al. Performance evaluation of Siemens ADVIA centaur and Roche MODULAR analytics E170 total 25-OH vitamin D assays. Clinical Biochemistry 2012;45:1485–90.

Please cite this article in press as: Farrell C-J, Herrmann M, Determination of vitamin D and its metabolites, Best Practice & Research Clinical Endocrinology & Metabolism (2013), http://dx.doi.org/10.1016/j.beem.2013.06.001

[62] Emmen JMA, Wielders JPM, Boer A-K, et al. The new Roche vitamin D total assay: fit for its purpose? Clincal Chemistry and Laboratory Medicine 2012;50:1969–72.
[63] Cluse ZN, Fudge AN, Whiting MJ, et al. Evaluation of 25-hydroxy vitamin D on the immunodiagnostic systems iSYS analyser. Annals of Clinical Biochemistry 2012;49:159–65.
[64] Horowitz GL, Remaley A. College of American pathologists ABVD proficiency survey discussion. Available at: http://www.cap.org/apps/docs/committees/chemistry/abvd-b_2012_discussion.pdf [accessed 21.02.13].
*[65] Carter G. Accuracy of 25-hydroxyvitamin D assays: confronting the issues. Current Drug Targets 2011;12:19–28.
[66] Cavalier E, Carlisi A, Bekaert A-C, et al. Human anti-animal interference in DiaSorin Liaison total 25(OH)-vitamin D assay: towards the end of a strange story? Clinica Chimica Acta 2012;413:537–8.
[67] Carter GD. 25-hydroxyvitamin D: a difficult analyte. Clinical Chemistry 2012;58:486–8.
[68] Gilbertson TJ, Stryd RP. High-performance liquid chromatographic assay for 25-hydroxyvitamin D3 in serum. Clinical Chemistry 1977;23:1700–4.
[69] Eisman JA, Shepard RM, DeLuca HF. Determitamin D2 and 25-hydroxyvitamin D3 in human plasma using high-pressure liquid chromatography. Analytical Biochemistry 1977;49:298–305.
[70] Shimada K, Mitamura K, Kitama N, Kawasaki M. Determination of 25-hydroxyvitamin D3in human plasma by reversed-phase high-performance liquid chromatography with ultraviolet detection. Journal of Chromatogratography 1977;689:409–14.
[71] Wootton A. Improving the measurement of 25-hydroxyvitamin D. Clinical Biochemist Reviews 2005;26:33–6.
[72] Hollis B. The determination of circulating 25-hydroxyvitamin D: no easy task. Journal of Clinical Endocrinology and Metabolism 2004;89:3149–51.
[73] Tai SS, Bedner M, Phinney KW. 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. Analytical Chemistry 2010;82:1942–8.
*[74] Stepman HC, Vanderroost A, Van Uytfanghe K, Thienpont LM. Candidate reference measurement procedures for serum 25-hydroxyvitamin D3 and 25-hydroxyvitamin D2 by using isotope-dilution liquid chromatography-tandem mass spectrometry. Clinical Chemistry 2011;57:441–8.
[75] Shah I, James R, Barker J, et al. Misleading measures in vitamin D analysis: a novel LC-MS/MS assay to account for epimers and isobars. Nutrition Journal 2011;10:46.
[76] Carter GD, Jones JG. Use of a common standard improves the performance of liquid chromatography-tandem mass spectrometry methods for serum 25-hydroxyvitmain-D. Annals of Clinical Biochemistry 2009;46:79–81.
[77] Netzel BC, Cradic KW, Bro ET, et al. Increasing liquid chromatography-tandem mass spectrometry throughput by mass tagging: a sample-multiplexed high-throughput assay for 25-hydroxyvitamin D2 and D3. Clinical Chemistry 2011;57:431–40.
[78] Al-Badr W, Martin KJ. Vitamin D and kidney disease. Clinical Journal of the American Society of Nephrology 2008;3:1555–60.
[79] Willnow TE, Nykjaer A. Pathways for kidney-specific uptake of the steroid hormone 25-hydroxyvitamin D3. Current Opinion in Lipidology 2002;13:255–60.
[80] Liberman UA, Halabe A, Samuel R, et al. End-organ resistance to 1,25-dihydroxycholecalciferol. Lancet 1980;3:504–6.
[81] Agus ZA. Hypercalcemia in granulomatous diseases. Available at: http://www.uptodate.com/contents/hypercalcemia-in-granulomatous-diseases?source=see_link [accessed 23.02.13].
[82] Brumbaugh PF, Haussler DH, Bressler R, Haussler MR. Radioreceptor assay for 1α,25-dihydroxyvitamin D3. Science 1974;183:1089–91.
[83] Reinhardt TA, Horst RL, Orf JW, Hollis BW. A microassay for 1,25-dihydroxyvitamin D notrequiring high performance liquid chromatography: application to clinical studies. Journal of Clinical Endocrinology and Metbolism 1984;58:91–8.
[84] Hollis BW, Kamerud JQ, Kurkowski A, et al. Quantification of circulating 1,25-dihydroxyvitamin D by radioimmunoassay with an 125I-labeled tracer. Clinical Chemistry 1996;42:586–92.
[85] Carter GD, Carter CR, Gunter E, et al. Measurement of Vitamin D metabolites: an international perspective on methodology and clinical interpretation. Journal Steroid Biochemistry and Molecular Biology 2004;89-90:467–71.
[86] Seiden-Long I, Vieth R. Evaluation of a 1,25-dihydoxyvitamin D enzyme immunoassay. Clinical Chemistry 2007;53:1104–8.
[87] Holick MF. Vitamin D status: measurement, interpretation and clinical application. Annals of Epidemiology 2009;19:73–8.
[88] Strathmann FG, Laha TJ, Hoofnagle AN. Quantification of 1alpha,25-dihydroxy vitamin D by immunoextraction and liquid chromatography-tandem mass spectrometry. Clinical Chemistry 2011;57:1279–85.
[89] Kissmeyer AM, Soone K. Sensitive analysis of 1alpha,25-dihydroxyvitamin D3 in biological fluids by liquid chromatography-tandem mass spectrometry. Journal of Chromatography A 2001;935:93–103.
[90] Aronov PA, Hall LM, Dettmer K. Metabolic profiling of major vitamin D metabolites using Diel-Alder derivatization and ultra-performance liquid chromatography-tandem mass spectrometry. Analyical and Bioanalytical Chemistry 2008;391:1917–30.
[91] Duan X, Weinstock-Guttman B, Wang H, et al. Ultrasensitive quantification of serum vitamin D metabolites using selective solid-phase extraction coupled to microflow liquid chromatography and isotope-dilution mass spectrometry. Analytical Chemistry 2010;82:2488–97.

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