



Vitamin D Deficiency: Causes & Treatments

Chapter 1

Vitamin D Testing in Clinical Settings: Methodologies, Accuracy and Standardization

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Keywords: Vitamin D; Accuracy; Standardization; Methodologies; Immuno-turbidimetry; Nanoparticles; EZ Vitamin D Assay; Diazyme.

Abbreviations: 25-OH Vitamin D: 25-hydroxy Vitamin D; VDBP: Vitamin D Binding Protein; 1,25-(OH)₂ Vitamin D: 1,25-dihydroxy Vitamin D; HPLC: High-Performance Liquid Chromatography; RIA: Radio ImmunoAssay; LC-MS/MS: Liquid Chromatography Tandem Mass Spectrometry; CLIA: Chemiluminescent ImmunoAssay; EIA: Enzyme ImmunoAssay; DEQAS: Vitamin D External Quality Assessment Scheme.

1. Introduction

Vitamin D is a group of liposoluble 9,10-seco steroids that includes vitamin D₂ (ergocalciferol) and vitamin D₃ (cholecalciferol) [1]. In humans, vitamin D₂ is acquired from food and vitamin supplements whereas vitamin D₃ is synthesized by the skin [1]. Both vitamin D₂ and vitamin D₃ are hydroxylated in the liver to produce 25-OH vitamin D which is further hydroxylated in the kidneys to produce 1,25-(OH)₂ vitamin D [2]. Whereas 1,25-(OH)₂ vitamin D is the physiologically active form of vitamin D, 25-OH vitamin D (the total amount of 25-OH vitamin D₂ and 25-OH vitamin D₃, also known as total 25-OH vitamin D) is the marker of the overall vitamin D status of a patient [3]. Vitamin D-related scientific research grew exponentially in the past few years, reaching more than 4000 yearly scientific publications since 2012. There is mounting scientific literature that links vitamin D deficiency to various health problems including bone metabolism disorders, cardiovascular diseases, cancer, autoimmune diseases, diabetes, infectious diseases and aging [4]. Accordingly, the clinical demand for vitamin D testing increased more than 10-fold in the past 10 years.

In 2011, the American Endocrine Society issued a clinical practice guideline on the evaluation, treatment, and prevention of vitamin D deficiency and recommended regular vitamin D screening for individuals at risk for deficiency [5]. In particular, the American Endocrine Society stated that patients suffering from obesity or osteoporosis as well as those receiving long-term corticosteroids treatments or anti-seizure medications are at high risk for vitamin D deficiency and should therefore be tested for vitamin D regularly. Accordingly, several US states expanded Medicare's coverage of vitamin D testing for all at-risk patients, including those with a Body Mass Index (BMI) of over 30. Based on the published literature, 25-OH vitamin D levels of less than 10 ng/mL are considered evidence of severe vitamin D deficiency [6]. Vitamin D levels in the 10-30 ng/ml range are a sign of insufficiency whereas levels in the 20-100 ng/mL range are a sign of vitamin D sufficiency [6]. Vitamin D levels over 100 ng/mL are considered toxic [6]. In the US, 30 ng/mL is used by most clinic laboratories as a cut-off for normal vitamin D results.

This book chapter is dedicated to the discussion of the various vitamin D testing methodologies and the current efforts aimed at harmonizing and standardizing the reported vitamin D results in clinical settings. The chapter starts by describing the analytical challenges that vitamin D represents for clinical chemists. It then proceeds with a survey of the current vitamin D testing methods. A special emphasis is given to Diazyme's two-reagent vitamin D assay (EZ Vitamin D Assay). An update on the accuracy of the various vitamin D assays used in clinical labs is presented and followed by an analysis and a discussion of the current harmonization and standardization efforts in the field of clinical vitamin D testing.

1.1. The Challenges of Vitamin D Testing

Accurate and precise measurement of the total concentration of 25-OH vitamin D is challenging for several reasons that include relatively low detection limit, the need to release the tightly bound vitamin D analyte(s) from their serum transporter, Vitamin D Binding Protein (VDBP), cross-reaction with a large collection of vitamin D metabolites and the lipophilicity of this peculiar analyte.

1.1.1. Lipophilicity of Vitamin D

As a steroid molecule, derivative of cholesterol, vitamin D is extremely lipophilic (i.e. very soluble in lipids) [7]. As a result, any assay measuring vitamin D may encounter some levels of interference from the wide variety of lipids that are found in biological fluids, such as serum and plasma. Typically, the released vitamin D fraction would partition between the aqueous and lipid fractions of serum or plasma. Since most modern immunoassays analyze the aqueous fraction of serum or plasma, the lipophilicity of vitamin D often leads to the underestimation of the true vitamin D concentration in the specimen.

Historically, the lipophilicity of vitamin D has been dealt with through the use of organic solvent extraction steps [8]. Although effective, this solution is only applicable to chromatographic methods and cannot be adapted to high throughput vitamin D testing methods, such as those used on automated chemistry analyzers. Vitamin D lipophilicity issues can now be counteracted by adding of a wide variety of traditional and proprietary detergents that act as emulsifiers, minimizing the partition of vitamin D between the hydrophilic and hydrophobic fractions of a biological fluid [8].

1.1.2. Vitamin D is Tightly Bound to its Serum Transporter

Because of its extreme lipophilicity, vitamin D cannot circulate as a free molecule in a highly aqueous environment such as blood. Instead, vitamin D circulates bound to a protein, Vitamin D Binding Protein (VDBP) [9]. Although the dissociation constants for the vitamin D/VDBP interaction vary between the different vitamin D metabolites, an average value of $2 \times 10^{(-9)}$ has been reported as an average K_d . This K_d , which is comparable to the K_d of most strong antigen-antibody interactions, shows that vitamin D is tightly bound to its transporter [9]. This renders impossible the direct quantification of vitamin D without sample treatment aimed at releasing vitamin D from its transporter.

The interaction between vitamin D and VDBP further complicates the assaying of vitamin D for two reasons. Firstly, VDBP is present in the blood in a much larger excess than vitamin D. It is estimated that more than 95% of VDBP found in blood circulates in an unbound form [9]. According to the law of mass action, this VDBP excess favors the formation of vitamin D/VDBP complexes and makes the quantification of vitamin D levels more challenging. Secondly, research studies have shown that the levels of VDBP vary significantly among patients. One study reported that VDBP levels can vary between 90 $\mu\text{g}/\text{mL}$ to as high as 1100 $\mu\text{g}/\text{mL}$ [10]. Different VDBP concentrations can affect the dissociation of vitamin D and subsequently its quantification. In addition to binding specifically to VDBP, vitamin D has also low affinity to several serum albumins (such as Human Serum Albumins, HSA), which rise the issue potential interference from the total amount of proteins found in serum (Total Protein).

Although vitamin D assaying methods now incorporate steps in which vitamin D is first released from its transporter, the general assumption is that the dissociation of vitamin D is quantitative (i.e. complete) and/or that this dissociation is uniform throughout a wide variety of patient specimen. In some instances, such assumptions have been proven wrong as experimental evidences of the contrary were reported in several immunoassays [11].

1.1.3. Specificity and Cross-reaction

Accurate assessment of the vitamin D status of an individual requires the measurement

of the sum of the concentration of 25-OH vitamin D2 and 25-OH vitamin D3 because there is no evidence of any physiological differences between these two forms of vitamin D [9]. As a result, any method assaying vitamin D must recognize 25-OH vitamin D2 and 25-OH vitamin D3 equally and must report the sum as total 25-OH vitamin D. Whereas this goal can be achieved relatively easily by chromatography and mass spectrometry-based methods (whereby 25-OH vitamin D2 and 25-OH vitamin D3 peaks can be resolved), it can be a real challenge for immunoassays (automated and non-automated). Indeed, because 25-OH vitamin D2 and 25-OH vitamin D3 have structural differences, have different binding affinities to VDBP and have different half-lives, it is possible that certain vitamin D assays recognize 25-OH vitamin D2 and 25-OH vitamin D3 differently and report inaccurate total 25-OH vitamin D results [13-14].

In addition to 25-OH vitamin D2 and 25-OH vitamin D3, there are over 40 different vitamin D metabolites that can potentially affect total vitamin D results reported by manufacturers of vitamin D assay [15]. Chiefly among the so-called vitamin D cross-reactants are 3-epi-25-OH vitamin D2 and 3-epi-25-OH vitamin D3. These epimers normally account for up to 8% of the total vitamin D of a patient and can in certain patient populations (such as infants and certain adults) be present at much higher levels [16-18]. In addition to 25-OH vitamin D epimers, other metabolites such as 24,25-(OH)₂ vitamin D often cross-react with vitamin D immunoassays and may need to be taken into account when reporting the total concentration of vitamin D [19].

1.1.4. Low Detection Limit

Typical limit of quantitation of commercially available vitamin D assays range between 4 and 10 ng/mL, which is relatively low compared to several other analytes measured in routine clinical chemistry. Accurately establishing the vitamin D status of a patient requires the measurement of low concentration of vitamin D, typically in the low ng/mL. The Institute of Medicine recommends a cut-off of 12 ng/mL to define a patient as deficient in vitamin D [20]. Because most patient populations were found to be deficient or insufficient in vitamin D, vitamin D reported results are often low and may represent a challenge to the sensitivity of certain immunoassays.

2. Vitamin D Testing Methods

Since the discovery of vitamin D in 1968, several methods have been developed to detect this peculiar analyte. The first competitive assay for vitamin D was developed in the early 1970s and opened the way for the rapid expansion of the vitamin D assaying landscape [19]. This expansion was supported along the way by an increasing body of scientific data

highlighting the importance of establishing the vitamin D status of a patient as a way to stay in good health and prevent diseases. As a result, vitamin D testing has increased exponentially in the past 15 years. In particular it grew by an average rate of 32% between 2014 and 2018 [8]. Below is brief survey of the major testing methods for 25-OH vitamin D.

2.1. Liquid Chromatography (LC)

One of the first published vitamin D detection methods (late 1970s) relied on the HPLC technique to resolve the various vitamin D metabolites and on UV absorption for detection and quantification [18]. Specimen are typically extracted using a mixture of organic solvents and separated by chromatography on, typically, reverse phase columns. Although the technique was cumbersome and very slow at the beginning (it took several hours to run just a few samples), a few companies now offer pseudo-automated versions of the method with a higher throughput. Samples still need to be first extracted manually, centrifuged and injected into the HPLC system, which dramatically increases experimental errors and raises reproducibility issues. Sample processing, injection and analysis can still take up to 37 min [15].

2.2. Liquid Chromatography in Tandem with Mass Spectrometry (LC-MS/MS)

The combination of liquid chromatography and mass spectrometry in the detection of 25-OH vitamin D offered a level of specificity and sensitivity that is unparalleled in the vitamin D testing realm. Technological advances in both sample preparation, chromatographic separation and mass spectrometry detection methods led to LC-MS/MS becoming the gold standard method in assaying vitamin D [19]. Not only the method can reliably detect very low concentrations of vitamin D (as low as 2 ng/mL), it can also report the individual concentrations of 25-OH vitamin D, 25-OH vitamin D₃ as well as the concentrations of their various epimers (3-epi-25-OH vitamin D). Furthermore, LC-MS/MS automation now allows high throughputs of up to 300 tests per hour [21].

Despite the apparent appeal of the LC-MS/MS method in vitamin D testing, it is important to point out that not all LC-MS/MS results being reported by clinical laboratories are equal in quality. There is indeed a wide variety of LC-MS/MS methods that dramatically differ in the way patient samples are prepared and extracted, the organic solvents used, the liquid chromatography method used and the mas-spectrometry detections methods employed. Whereas Isotope Dilution represents the best LC-MS/MS method used to date for assaying vitamin D, there are several LC-MS/MS methods locally developed and validated by clinical laboratories (the so-called “home-brew” LC-MS/MS assays) that are sub-standard in terms of sensitivity, specificity and accuracy. As such, LC-MS/MS vitamin D results should only be considered reliable if they use the very specific state-of-the-art Isotope Dilution method [19].

2.3. Radio-Immunoassays (RIA)

Radio-Immunoassays applied to vitamin D testing has been in use for several decades. After a pre-treatment step, during which vitamin D metabolites are released from their serum transporter, a known amount of a tracer (25-OH vitamin D labeled with the radioactive ^{125}I) competes with the patient's 25-OH vitamin D2 and 25-OH vitamin D3 for a monoclonal antibody specific to 25-OH vitamin D (typically immobilized on a solid phase). Following a lengthy incubation (1-3 hours) and a few washing steps, the amount of radioactivity associated with the mobile phase is quantified and correlated to the concentration of vitamin D in the patient's serum, in an inversely proportional manner. Although RIA methods offer excellent sensitivity, they are still cumbersome and have low throughput. In addition, like any other immunoassays, RIA highly depends on the quality of the monoclonal antibody used (affinity and specificity, among other).

2.4. Enzyme Immunoassays (EIA) and Enzyme-Linked Immunosorbent Assays (ELISA)

EIA and ELISA applied to vitamin D testing share several features with RIA that include the use of antibodies and solid phases. In fact, the first EIA and ELISA assays developed in the 1970s were specifically designed to mimic the RIA method while bypassing the use of radioactive elements. In-lieu of quantifying the amount of a radioactive label, EIA and ELISA quantify the photometric signal generated after the enzymatic transformation of a chromogenic substrate. Detailed description of the EIA and ELISA technology can be found elsewhere [22]. Whether they are indirect, competitive or sandwich, various EIA/ELISA assays are currently used to measure total 25-OH vitamin D. Although a lot of progress has been made to improve the throughput of these assays, the results reported by these assays still suffer from high variability that is related to the use of different sample treatment/extraction methods and/or antibody with different specificities [22].

2.5. Chemiluminescence Immunoassays (CLIA)

CLIA applied to the detection of vitamin D is a variation of EIA in which a labelled antibody induces the transformation of a substrate into a photon-emitting molecule. Likewise EIA and ELISA, whereby a substrate is transformed into a colored molecule measured spectrophotometrically, CLIA measures the amount of light emitted and correlates it to the amount of vitamin D [20]. CLIA applied to vitamin D brought several benefits that include ultra-sensitivity, wider Assay Measuring Range (AMR), wider linearity range and suitability for automation. Furthermore, because CLIA involves a solid phase (magnetic beads coated with antibodies or antigens) that can be simply washed by magnetic pull down, the assay is less sensitive to interfering substances and to the so-called matrix effects observed in other assays.

CLIA-based vitamin D assays grew in popularity in the last decade, with all major manufacturers of medical devices producing and distributing their own versions of CLIA for vitamin D. Whereas the advantages of such assays cannot be dismissed, CLIA still requires specific instruments (often different from those used in general clinical chemistry) and still have a relatively low throughput as most CLIA vitamin D tests take up to 40 min to complete. These limitations add to antibodies-related specificity issues that most vitamin D-based immunoassays suffer from.

2.6. First Generation Vitamin D Assay for General Chemistry

Despite the past years' exponential progress and the diversification of the technologies of vitamin D detection, vitamin D testing still remained a specialty test that can be run only on a specific list of automated analyzers with specific liquid handling and detection capabilities (mostly chemiluminescent).

A few years ago, Diazyme Laboratories, Inc. (a company located in Poway, CA) initiated the project of developing the next generation of vitamin D assays that can be run on ubiquitous general chemistry analyzers, using the ubiquitous photometric detection methods. In executing this ambitious project, Diazyme Laboratories' goal was to "democratize" vitamin D testing by providing vitamin D testing capabilities to virtually any clinical chemistry laboratory with a standard general chemistry analyzer.

In 2013, Diazyme Laboratories released its first fully automated enzymatic assay for general chemistry analyzers (Diazyme 25-OH Vitamin D Assay, FDA reference k133410). The assay used the Cloned-Enzyme Donor Immuno-Assay (CEDIA) technology [8]. It is based on the enzymatic principle of α -complementation of the enzyme β -galactosidase, whereby a small portion of the enzyme (the so-called enzyme donor) is chemically linked to a vitamin D moiety and used to modulate the complementation of a larger portion of the enzyme (the so-called enzyme acceptor), in the presence of an anti-vitamin D antibody and the vitamin D analyte found in the patient's sample [24]. Upon the sequential addition of three-reagents, a photometric signal is generated and is proportional to the vitamin D concentration of a specimen. Detailed description of this assay and its performance can be found elsewhere [8]. This first fully automated assay for general chemistry can be run on clinical analyzers capable of handling a diluent and three reagents as well as a reagent incubation scheme of 20 min. Although the assay did not match the speed and simplicity the fastest general chemistry assays (up to two reagents, with results appearing within 5 to 10 minutes), by bringing for the first time vitamin D testing to the general chemistry platform, this assay represented a new milestone in the testing of vitamin D.

2.7. Second Generation Vitamin D Assay for General Chemistry

Following on the footsteps of its first generation homogenous assay for vitamin D, Diazyme Laboratories released in 2018 its second generation vitamin D assay for general chemistry, under the trade name EZ Vitamin D Assay. The assay which was cleared by the FDA on 01/11/2018 (k172992) brought several improvements over its first generation version. The new assay is nano-particle based, liquid stable, uses only two reagents and reports results in 10 min or less.

Briefly, the assay's methodology is based on a modification of the latex-enhanced immune-turbidimetric technique. The assay uses a proprietary pair of antibodies that are conjugated to latex particles. The first antibody binds directly to 25-OH vitamin D, forming a complex that is recognized by the second antibody. The iteration of these binding events triggers the agglutination of latex particles and the generation of turbidimetric signal that can be quantified photometrically. Detailed description of the assay and its performance can be found elsewhere [25].

By eliminating sample dilution and significantly reducing incubation times, the assay has, to the best of our knowledge, the highest throughput of any vitamin D assay currently on the market (over 500 tests per hour on c701). The assay has now been validated on a wide variety of traditional clinical chemistry analyzers that includes Beckman's AU680, Roche's Modular P, Roche's Cobas c501, Roche's Cobas c701, Roche's Hitachi 917, Diazyme's c270, Abbott's Architect c4000, Abbott's Architect c6000 and Horiba's Pentra 400.

3. An Update on the Accuracy of Vitamin D Assays

3.1. The Vitamin D Accuracy Debate

By definition, the accuracy of an experimental result is a measure of its closeness to the true, or accepted, value. Establishing the accuracy of a vitamin D test result is a uniquely challenging problem for several reasons.

Firstly, for a given vitamin D specimen there must be a true/accepted value. This means that there should be a consensus among the clinical chemistry community about a specific method as being the one providing the true, or at least the widely accepted value. Although most clinicians would argue that LC-MS/MS reports the true/acceptable value of a vitamin D specimen, not all LC-MS/MS methods are equal because they dramatically differ in the way samples are pre-treated for liquid chromatography and the way samples are analyzed and detected by mass spectrometry. As explained in paragraph 2.2, the Isotope Dilution LC-MS/MS is widely accepted as the state-of-the-art detection method for vitamin D. Results reported by this method should be considered as the true/accepted value. This method is used by the Na-

tional Institute of Standards and Technology (NIST), the Center for Disease Control and Prevention (CDC), and Ghent University. Several of the “home-brew” LC-MS/MS used across the US do not use Isotope Dilution and may not report accurate results. As a result the scarcity of vitamin D samples assigned by Isotope Dilution LC-MS/MS is a major problem in the vitamin D accuracy debate.

Secondly, the accuracy of vitamin D results is ultimately used to establish a clinical diagnostic (deficient, insufficient, sufficient, possible harm). Yet, as of 2018, there is still no universal standard that establishes the cut-off for these various diagnostic levels. The Institute of Medicine (IOM) defines the deficient level as < 12 ng/mL whereas the Endocrine Society (ES) defines it as < 20 ng/mL. Insufficient is defined as 12-20 ng/mL by IOM whereas ES defines it as 21-29 ng/mL [19]. Sufficient is defined as 20-30 ng/mL by IOM whereas ES defines it as 30-100 ng/mL. Possible harm is defined as > 50 ng/mL by IOM whereas ES defines it as > 100 ng/mL [19]. As a result, discussing the accuracy of vitamin D results in terms of reporting the true/accepted clinical diagnostic is challenging because cut-offs vary from a clinical authority institution to another.

3.2. A 2018 Snapshot of the Accuracy of Various Vitamin D Methods

In this section, we wanted to report on the current (2017-2018) accuracy status of various vitamin D methodologies. Presenting various linear regression plots of method Y versus method X and assessing slope, bias and R² is not the best way to establish accuracy because these linear regression parameters are strongly biased by the number of samples used, the range of value measured and the dispersion of data points around the identity line.

We hypothesized that the best way to assess the accuracy of the vitamin D testing realm is to have a set of unaltered samples measured by the Isotope Dilution LC-MS/MS method and have to obtained results compared to those reported by various commercial vitamin D assays (including various LC-MS/MS methods) in term of % bias from the true value (i.e. the Isotope Dilution LC-MS/MS value). The CDC’s Vitamin D Standardization-Certification Program (VDSCP) updated published data (September 2018) provides exactly the kind of data that we are looking for. It, indeed, reports on the “Individual samples pass rate” of each vitamin D method as the percentage of individual samples (out of a set of 40) that met certification criteria of mean bias of $\pm 5\%$ versus the CDC’s Isotope Dilution LC-MS/MS value. Results are shown in Table 1.

Three major points stand out from this table. Firstly, LC-MS/MS methods remain the most accurate method for assaying vitamin D as their individual pass rate is consistently higher than 50%. Secondly, as expected, there is a wide variation in the accuracy of different LC-MS/MS methods as individual pass rates range between 53% and 90%. This is due to the variation

in sample treatment and sample detection methods as explained in paragraph 2.2. Thirdly, immunoassays (whether CLIA, EIA, or competitive binding assay) have consistently low pass individual pass rates (less than 50%). Some methods had as low of a passing rate as 23%. As a whole, table 1 shows that as of 2018, vitamin D are still in need of significant improvement in accuracy to match the reference method of Isotope Dilution LC-MS/MS.

Method	Principle	2018 Individual Samples Pass Rate (%)	2017 Individual Samples Pass Rate (%)
ARUP 25-Hydroxyvitamin D2 and D3 by Tandem Mass Spectrometry, Serum	LC-MS/MS	53	58
CCR Laboratory University of Maryland Clinical Pathology Total 25 OH-Vitamin D	LC-MS/MS	65	68
Cork Centre for Vitamin D and Nutrition Research Total serum 25OHD	LC-MS/MS	68	85
Covance Central Laboratory Services, Inc, Total 25 Hydroxy-Vitamin D2/D3	LC-MS/MS	70	65
Nutrition Research Division Health Canada Serum Total 25OHD, 25OHD3, 25OHD2	LC-MS/MS	73	77
SCIEX Diagnostics Total 25-HydroxyvitaminD (Vitamin D2/Vitamin D3) II	LC-MS/MS	90	na
University of Minnesota MEBRL 25-Hydroxy Vitamin D2 and D3	LC-MS/MS	na	78
DiaSorin LIAISON® 25 OH Vitamin D TOTAL Assay	CLIA	na	38
Immunodiagnostic Systems (IDS) IDS-iSYS 25 VitD S (IS-2500)	CLIA	40	45
Siemens Healthcare Diagnostics ADVIA Centaur® Vitamin D Total assay	CLIA	28	25
Siemens Healthcare Diagnostics Dimension®ExL™ Vitamin D Total assay	CLIA	40	23
Abbott GmbH ARCHITECT 25-OH Vitamin D	CLIA	28	38
Immunodiagnostic Systems (IDS) 25-Hydroxy Vitamin D EIA	EIA	25	25
Tosoh Corporation ST AIA-PACK 25-OH Vitamin D	EIA	na	30
Roche Diagnostics GmbH Elecsys Vitamin D total II	ECL binding assay	na	43

Table 1: 2018 and 2017 Individual passing rate (bias less than 5%) for a set of 40 samples run with various vitamin D methods as part of the CDC VDSCP program.

Diazyme enrolled into the VDSCP program in 2018 and results will be published in early 2019. Data retrieved on September 28th, 2018 from:

https://www.cdc.gov/labstandards/pdf/hs/CDC_Certified_Vitamin_D_Procedures-508.pdf

One could argue that the low individual samples passing rates reported in table 1 could

due to the fact that immunoassays (CLIA, EIA and the like) are significantly different in terms of methodology from LC-MS/MS and as a result should only be compared to their respective peer-methods (i.e. other immunoassays). Table 2 list 10 samples that have been assayed with leading vitamin D immunoassays currently in the US market as part of a research study published in 2018 [26]. Figure 1 shows a histogram plot of 4 representative samples.

Serum	Abbott Architect	Roche Elecsys	Siemens Centaur	Ortho Vitros	Diasorin Liaison	IDS SYS	Beckman 600/800	All Mean	All STDEV	CV
1	37.0	24.2	57.9	61.8	37.9	47.0	33.8	42.8	13.5	31.5%
2	48.5	31.6	80.4	74.5	50.0	57.0	46.3	55.5	16.9	30.5%
3	24.4	14.7	50.3	78.2	50.5	42.5	38.2	42.7	20.5	48.0%
4	12.5	9.3	36.8	82.0	37.0	23.5	31.0	33.2	24.2	72.9%
5	30.8	15.3	54.1	67.2	34.3	30.0	32.5	37.7	17.3	45.7%
6	47.3	34.5	73.4	68.5	53.1	50.5	49.3	53.8	13.2	24.5%
7	55.8	44.5	87.9	78.7	63.5	57.0	53.0	62.9	15.3	24.3%
8	39.9	28.8	60.8	53.5	40.8	38.0	33.5	42.2	11.2	26.6%
9	41.3	23.8	66.1	72.1	51.2	44.0	44.3	49.0	16.2	33.1%
10	28.6	17.3	53.4	63.0	37.0	33.0	30.6	37.6	15.6	41.5%

Table 2: Serum vitamin D levels (nmol/L) of 10 samples tested with seven leading immunoassays. Mean of all methods, standard deviation of all methods (STDEV) and coefficient of variance of all methods (CV) are also reported.

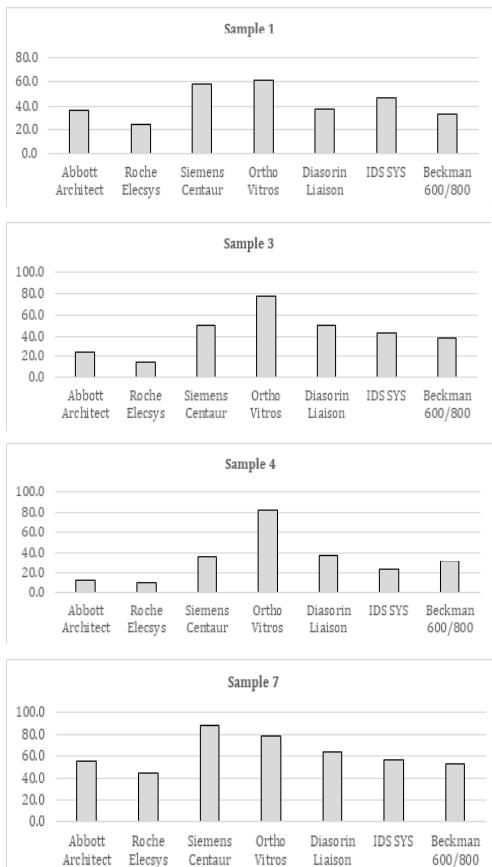


Figure 1: Histogram plot of representative samples 1, 3, 4 and 7 from table 2.

Results shown in Table 2 and Figure 1 show that agreement between immuno-assays is still poor with coefficients of variation among the 7 listed vitamin D methods ranging between 24.3% and 72.9%. Similar conclusions about the high variability of vitamin D results has been reported in multiple recent research studies [27-35]. Interestingly, Table 2 and Figure 1 show that for a given method, the reported result is not consistently higher or consistently lower than the mean of all methods. In fact, it is sample dependent. This suggests that the discrepancies seen between the different methods may not be solely related to calibrator traceability (i.e. may not be completely eliminated by simply adjusting calibrators' assigned values). We believe that the disagreement between the different vitamin D methods is related to differences in the interference profiles of these assays and differences in the specificity of the antibodies used.

3.3 The Interference Problem

LC-MS/MS techniques remove most of the component of the vitamin D sample matrix in order to detect and quantify 25-OH vitamin D. This is typically achieved through sample extractions using organic solvents. Since the handling of organic solvents and dual phases (solid and liquid) is not feasible in automated chemistry analyzers, automated immunoassays have to rely on “mild” conditions to dissociate vitamin D from its transporter. This is typically achieved by lowering the pH of the sample. Whereas this method releases vitamin D metabolites and makes them available for measurement, it leaves most of the components of the sample’s matrix unchanged and susceptible to interfere with the measurement. A key interfering substance is VDBP which is found in large excess in serum and the excess of which varies from patient to patient [27-35].

3.4 The Cross-reaction Problem

Because of their reliance on antibodies, the results reported by vitamin D immunoassays are strongly correlated to the specificity of the antibodies used and their cross-reaction to various vitamin D metabolites.

One of the major cross-reaction problem seen in vitamin D assays is the relative cross-reaction between 25-OH vitamin D₂ and 25-OH vitamin D₃. In reporting total 25-OH vitamin D (the sum of 25-OH vitamin D₂ and 25-OH vitamin D₃), an immunoassay must ideally recognize, equally, 25-OH vitamin D₂ and 25-OH vitamin D₃. Although manufacturers of vitamin D immunoassays often claim in their package that their assays recognize the two metabolites equally, some of the manufacturers fail to detect endogenous 25-OH vitamin D₂ (i.e. 25-OH vitamin D₂ in a patient serum sample, acquired through dietary intakes) [33]. As a result, several on-the-market vitamin D assays underestimate the total 25-OH vitamin D concentration by failing to measure a fraction or all of the endogenous 25-OH vitamin D₂ metabolites. The 25-OH vitamin D₂ cross-reaction problem is puzzling for two reasons. Firstly,

it appears that some immunoassays have different cross-reaction profiles for 25-OH vitamin D2. Whereas exogenous 25-OH vitamin D2 (i.e. pure vitamin D2 spiked into a serum sample) often cross-reacts at a nearly 100% rate to 25-OH vitamin D3, cross-reaction to endogenous 25-OH vitamin D2 is often not 100%. Secondly, the fraction of endogenous 25-OH vitamin D2 detected by immunoassays can vary from patient sample to patient sample [33]. It appears that there might be a peculiar chemical feature associated with endogenous 25-OH vitamin D2 that is still poorly understood.

Another cross-reaction problem associated with immunoassays is that related to 3-epi-25-OH vitamin D and 24,25-(OH)₂-vitamin D. Early scientific literature suggested that levels of 3-epi-25-OH vitamin D were low in adults, and therefore should not be significantly affecting the results reported various immunoassays. However, more recent scientific data shows that the levels of 3-epi-25-OH vitamin D can represent up to 17% of the total 25-OH vitamin D [30]. Similarly, the levels of 24,25-(OH)₂ vitamin D, initially thought to be low in adults, can be as high as 5.4 ng/mL [30].

It is important to note that the issue of cross-reaction is not exclusive to immunoassays. Because various assays can drastically differ in their ability to resolve the peaks of 3-epi-25-OH vitamin D and/or 24,25-(OH)₂ vitamin D, chromatography-based and mass spectrometry-based vitamin D assays can also report results that are modulated by the concentrations of 3-epi-25-OH vitamin D and 24,25-(OH)₂ vitamin D.

By taking into account the different 25-OH vitamin D2 cross-reaction levels, 3-epi-25-OH vitamin D levels of up to 17% of the total vitamin D and 24,25-(OH)₂ vitamin D levels of up to 5.4 ng/mL, it is easy to understand why various vitamin D assays often poorly correlate to each other.

4. Standardization, Traceability and Harmonization of Vitamin D Results

4.1 Definitions

Before discussing the current efforts of standardization, traceability and harmonization of vitamin D results, it is important to clearly define these terms that are often confused with one another in the clinical chemistry community.

By definition, standardized vitamin D measurements are measurements that accurately match (within established experimental errors) the Isotope Dilution LC-MS/MS measurements reported by NIST, Ghent University or the CDC [19]. These three methods establish the standards for vitamin D because they conform to the International Organization for Standardization requirements [19]. As such, standardization refers to reporting results that accurately

match one or multiple “gold-standard” methods.

Traceability is simply a tool used to achieve standardization. Traceability works by establishing a continuous chain of procedures that connects the analyte being measured to the values reported by the gold standard method(s). In the case of vitamin D, this can take the form of manufacturing master calibrators the values of which are established by the gold standard method, then making production calibrators that are traceable to the master calibrators and finally reporting vitamin D result by matching the activity of a given specimen to the activity of the production calibrators. Because each of the traceability steps brings experimental errors of its own, there is an increased deviation from the gold standard down the traceability chain. Deviation starts with the establishment of master calibrators and ends with the reporting of the vitamin D result of a patient sample. Sample matrix, interference profiles and cross-reaction profiles tremendously affect the reliability of the traceability chain.

Harmonization is the reporting by various laboratories of the same vitamin D results, regardless of their accuracy. This means that two vitamin D methods are considered harmonized if they report the same results, even if these results are not the true values for the samples tested. Harmonization and standardization differ in the fact that standardization is a specific harmonization in which results match the true value. As such, one can make the statement that if all vitamin D methods were to be standardized, these methods will automatically become harmonized. By contrast, harmonizing vitamin D methods, does not necessarily standardize them. Whereas vitamin D results ideally need to be standardized, it is common for clinical chemistry laboratory to only require a given method to be harmonized to another given method, at the expense of accuracy.

4.2. Standardization Initiatives

4.2.1. The NIST Vitamin D Reference Materials

The NIST Standard Reference Material (SRM) for vitamin D was first introduced by NIST on July 14th, 2009. This SRM (identified as 972 then 972a) consists of four blood serum sample pools (level 1 to level 4) with certified values for 25-OH vitamin D₂, 25-OH vitamin D₃ and 3-epi-25-OH vitamin D₃. NIST SRM972a is essential in the establishment of production calibrators as part of the traceability chain. In addition to SRM972a, NIST provides SRM2972a which consists of stock solutions of 25-OH vitamin D that can be used to prepare calibrators. The importance of NIST vitamin D SRM materials is further highlighted by the reporting of the separate levels of 25-OH vitamin D₂, 3-epi-25-OH vitamin D₃ and 24,25-(OH)₂ vitamin D₃ which can help in dealing with the cross-reaction issues discussed in section 3.3. Currently, SRM972a highest level measures a total of ~ 33 ng/mL of 25-OH vitamin D. Going forward, it would be helpful if NIST could provide additional levels to cover the dynamic

range of most vitamin D assays (typically up to ~150 ng/mL).

4.2.2. The CDC Vitamin D Standardization-Certification Program (VDSCP)

In order to improve the accuracy and the robustness of various vitamin D tests, the CDC introduced a certification program in which participants are provided with a blind panel of single-donor serum samples for which they are assessed for both imprecision and bias compared to the CDC's reference method for testing vitamin D (Isotope Dilution LC-MS/MS).

The co-called VDSCP program is comprised of two phases. During the first phase, participants are provided with a panel of vitamin D samples with assigned values. The CDC recommends to run a minimum of 40 samples and to process the method comparison data according to the Clinical and Laboratory Standards Institute (CLSI) Protocol EP9-A, "Method comparison and bias estimation using patient samples". These samples are used to assess the participant's initial performance and to make adjustment to the device's reagents and/or calibrators when needed. During the second phase, participants are enrolled in the actual certification program. During the certification process, participants receive, quarterly, a set of 10 blind samples for which they have to report vitamin D measured values as well as the level of imprecision. Once the data is received and processed by the CDC, participants receive reports detailing their performance for each quarterly sample set in term of bias to the CDC's reference method as well as total imprecision. The CDC considers that a participant satisfies the certification requirement if the mean bias for all samples tested is less than 5% and if the total sample imprecision is $\leq 10\%$. The list of certified participants is published by the CDC along with their individual passing rate (percent of samples out of 40 that had a bias of less than 5%).

Although there is a general consensus among the vitamin D community about the importance of the CDC's VDSCP, the use of mean bias to grade the performance of participants may not be the best way to reliably assess accuracy. Indeed, a given set of 40 samples can still have a mean bias of less than 5% even if the bias for each individual sample is significantly higher than the 5% limit [16]. In addition, the 5% bias criterion for individual samples might be too stringent and difficult to achieve by most vitamin D because it falls within the range of imprecision of the assay itself, especially if samples have low vitamin D concentrations. A better way to grade the performance of each participant could be setting a passing criterion for each sample at $\pm 15\%$ bias from the CDC's reference method and an overall individual passing rate (percent of samples out of 40 with a bias of $\pm 15\%$) to 85%. As the performance of various vitamin D assays improves over the years, the passing criteria could be updated to 10% individual bias and 90% individual passing rate.

4.2.3. Vitamin D Proficiency Testing (PT) Programs

Proficiency testing (PT), which initially started as a voluntary clinical laboratory practice, is increasingly becoming important for all clinical labs in the US and elsewhere. The PT scheme is similar to the CDC's VDSCP in the sense that it involves the receipt by participants of blind vitamin D samples (quarterly or semi-annually) and the "grading" of the reported results. PT differs, however, from VDSCP in the sense that the number of yearly blind samples tested is smaller (typically 20 or less). Although several PT program for vitamin D have been introduced over the past few years, two particular programs are, in our opinion, the most reliable: The Vitamin D External Quality Assessment Scheme (DEQAS) and the College of American Pathologists Accuracy-Based Vitamin D (CAP-ABVD).

The UK-based DEQAS PT program is by far the oldest and most established vitamin D PT program. This program was introduced in 1989 and grew to an international program with over 1200 participants in over 54 countries (including the US). DEQAS involves the quarterly assessment of a set of 5 unprocessed serum samples. The first 4 samples are used for assessment whereas the fifth sample is often used for research purposes. NIST reference values for these samples are published and the performance of each participant is evaluated in term of % bias from the mean of all methods. Typical acceptance criterion is set at $\pm 25\%$. It is important to note that DEQAS is recognized by the College of American Pathologists (CAP) as a PT program in the US. A key important feature of the DEQAS program is that it provides participants with unaltered serum samples, containing no preservatives. No spiking of exogenous substances or dilutions are used in the DEQAS scheme. As such, DEQAS mitigates the so-called matrix effect and provides participants with samples that faithfully replicate the behavior of real clinical samples. Because of its success, the DEQAS program needs to prepare large volumes of serum to distribute to all of its participants. Such volumes cannot possibly be obtained from single donors. As such, each DEQAS sample is typically a pool of a few single donors. To pass the certification requirements, DEQAS participants must have an individual sample passing rate of at least 75%. This means that at least 16 out of the 20 samples tested yearly must have values falling within $\pm 25\%$ of the NIST reference value.

The CAP-ABVD program consists of two yearly shipments (namely A and B). Each shipment contains three blind samples to be measured by each participant. CAP-ABVD samples are unaltered serum samples obtained from pools of individual donors. It is important to note that the CAP-ABVD program is exclusively dedicated to vitamin D and is different from a separate multi-analyte PT survey that includes vitamin D and that is also offered by CAP. An interesting feature of the CAP-ABVD program is that it grades the performance of its participants by peer groups (labs using similar methodologies). Likewise DEQAS, CAP-ABVD provides LC-MS/MS reference values for each of its samples, allowing participants to assess their

performance vis-à-vis of their peers as well as the gold standard method. As such, CAP-ABVD not only contributes to the harmonization of vitamin D results among peer groups, it also significantly contributes to overall vitamin D standardization efforts by inviting manufacturers of vitamin D measuring devices to adjust reagents and/or calibrators to improve accuracy.

5. Conclusion

Vitamin D is a steroid molecule that plays an essential role in the metabolism. A large body of scientific literature has linked deficiency in 25-OH vitamin D (the circulating form of vitamin D) to rickets, osteoporosis and increased risks for cardiovascular diseases, certain forms of cancers and certain mental health issues. As a result, measuring the levels of 25-OH vitamin D gained the attention of the medical and the clinical chemistry communities. Several methodologies have been developed to measure the levels of 25-OH vitamin D in human serum and plasma. As early as the 1970s, clinicians were able to measure the levels of 25-OH vitamin D using ELISA and RIA. Over the years, LC, LC-MS, CLIA and other EIA methods were developed. Faced with the exponential growth of the 25-OH vitamin D testing market, manufacturers had to develop fast and automated assays. Diazyme's first enzymatic vitamin D assay for general chemistry analyzers (released in 2013) as well as Diazyme's first nanoparticle-enhanced, two-reagent vitamin D assay for general chemistry (released in 2018) represent major milestones in the "democratization" of vitamin D testing.

With the development of a large array of vitamin D testing methodologies, accuracy and harmonization issues started to appear and the need for standardization and traceability became urgent. Firstly, a gold standard reference method for 25-OH vitamin D was established and consisted of the Isotope Dilution LC-MS/MS method. This technology is available at NIST, the CDC and Ghent University. Secondly, reference vitamin D material assigned with the method described above was made available by NIST (SRM972a) to help manufacturers of vitamin D assays trace their calibrators to the reference method. Thirdly, a certification program involving 40 samples assigned with the reference method was introduced by the CDC (VDSCP) to improve the traceability of various methods to the reference method. Finally, various proficiency testing programs (mainly DEQAS and CAP-ABVD) were introduced to assess the quality and accuracy of the final vitamin D results reported by clinical laboratories.

Thanks to the various traceability tools provided mainly by NIST and the CDC, standardization levels (as well as harmonization levels) improved in the past few years. There is, however, still a lot of work to do to achieve acceptable standardization levels across the realm of vitamin D testing. It appears that most vitamin D methods have now calibrators that are traceable to the reference method. This means that a linear regression of the comparison data between a given vitamin D method and the reference vitamin D method is likely to produce

a slope that is statistically close to 1.0. However, the scatter along this identity line is still significant for almost all vitamin D methods, when compared to the reference method. In our opinion, this is due to issues unrelated to standardization but rather to the inner properties of each vitamin D assay. We believe that differences in the interference and cross-reaction profiles of the antibodies used in various immunoassays as well as the different susceptibilities to the complex human serum matrix still account for most of the between-assay variability in the reported vitamin D results. Harmonization of the human serum treatment methods as well as the narrowing of the spectrum of vitamin D antibodies used are, in our opinion, the future critical steps to undertake in order to bring the various vitamin D testing methods to a universal agreement.

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