Santiago et al. Scr Med. 2025 Sep-Oct;56(5):857-69. DOI:10.5937/scriptamed56-61645

ORIGINAL ARTICLE

Evaluation of a Newly Developed LC-MS/MS Vitamin D Assay

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Abstract

Background/Aim: Globally, deficiency of vitamin D is highly prevalent. Besides the known consequences of vitamin D deficiency to bone health, there is now strong evidence that links low vitamin D status to an increase in the risk for diabetes, cancer, cardiovascular disease and autoimmune diseases. It is therefore important to have a highly accurate, reproducible and cost-effective test that is highly predictive of vitamin D status and of diagnostic value. This study was undertaken to validate a newly developed high throughput liquid chromatography with tandem mass spectrometry (LC-MS/MS) 25-hydroxy vitamin D (25(OH)D) assay against current gold standard assays measured at two independent reference laboratories.
Methods: The initial study (n = 40) and follow up study (n = 40) recruited healthy adult men and women volunteers (18 to 55 years old). Vitamin D (25(OH)D) was measured using a targeted LC–MS/MS method.
Results: Unexpectedly, data were not consistent with the values for 25(OH)D obtained from the two independent reference laboratories (as evidenced by correlation coefficients and Bland Altman analyses), although the results between the two reference laboratories were in agreement and highly correlated.
Conclusion: These findings highlight the continued efforts and needs for harmonisation of results and standardisation of analytical methods for 25(OH)D for diagnostic accuracy.

Key words: Vitamin D; Cholecalciferol; Analytical methods; Liquid chromatography with tandem mass spectrometry; Clinical application; Diagnostic value.

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Citation:
Santiago JJ, Mitchell RW, Hunt W, Tappia PS, Scantlebury DC, Ramjiawan B. Evaluation of a newly developed LC-MS/MS vitamin D assay. Scr Med. 2025 Sep-Oct;56(5):857-69.

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Received: 22 September 2025
Accepted: 19 October 2025

Introduction

Vitamin D deficiency is a common global health problem that is estimated to affect approximately 1 billion people across the world with 30 - 50 % of the global population considered to be vitamin D insufficient.[1-5] Although the main function of vitamin D is in bone and mineral metabolism, evidence is now suggestive of vitamin D deficiency, to be either a causative or associated characteristic in several different pathological conditions such as kidney disease, parathyroid dysfunction, sarcoidosis, rickets[6] and rheumatoid arthritis.[7] Furthermore, some studies have now revealed that vitamin D status may have an important physiological and pathophysiological role in different diseases such as cardiovascular disease, diabetes, obesity and some cancers.[8-17] However,

other studies have revealed inconsistencies and inconclusive findings on the health effects.[18] This could be related to how vitamin D status is determined and its interpretation. Thus, the accurate analysis of vitamin D and related metabolites is a challenge for analytical laboratories and therefore establishing an optimal assay method for measuring circulating levels of vitamin D is a matter of continuous deliberation.[19] Since 25(OH) D has a half-life of approximately 15 days[20] the measurement of serum 25(OH)D is the current standard for assessing vitamin D status. However, there is no standardisation in place regarding methodology and techniques for measuring 25(OH)D.[21] In addition, there are challenges in the extraction as well as in the ability to measure vitamin D and interfering metabolites[22] and thus large variability exists among the assays used.[23, 24]

Accordingly, it is of particular importance for analytical laboratories to be aware of the performance as well as the limitations of their vitamin D assay systems and to ensure reliability in the measurement of vitamin D.[25] Furthermore, there are several aspects that need to be considered when determining the accuracy of vitamin D measurements including adverse biological matrix effects, derivatisation reactions, influence of ionisation sources, contribution of epimers, as well as standardisation of assays between laboratories.[26] Indeed, standardisation or harmonisation of the different laboratory procedures for measuring 25(OH)D is of importance to ensure clinical relevance and diagnostic value when assessing vitamin D status of an individual.[27] However, despite standardisation efforts, assay variations and challenges remain, particularly with respect to specific patient groups.[28] The present study was undertaken to validate our newly developed assay (Test Lab) as well as to determine whether a correlation exists between our assay for vitamin D and the current standard assay for Vitamin D as measured by two independent laboratories (Reference Lab 1 and Reference Lab 2). It was also envisioned that presented assay may be utilised to establish a reference range in the normal healthy population. Thus, the health status of the study population as well as the presence of serum confounding factors that may interfere with the 25(OH)D assay were also determined.

Methods

Study design and participant selection

The Biomedical Research Ethics Board of the University of Manitoba and the Research Review Committee of the St Boniface Hospital approved this study. The initial study (n = 40) and follow up study (n = 40) recruited healthy adult men and women volunteers (18 to 55 years old). Potential participants were recruited by study advertisement and each participant was screened for eligibility. All volunteers enrolled into the study consented prior to participation and were then screened using a two-stage method. The first stage was a questionnaire regarding health history, demographics and activity/fitness levels. Individuals with no reported medical issues proceeded to the second stage of eligibility screening. Participants were asked to provide a urine sample for analysis of various parameters of general health. Eligible participants were individuals that exhibited normal levels as described by the test strip parameters. Enrolled participants were then asked to provide a non-fasted blood sample for analysis. The serum was also tested for glycosylated haemoglobin (HbA_1c); high-density lipoprotein (HDL), low-density lipoprotein (LDL) and total cholesterol, triglycerides as well as the inflammatory marker, C-reactive protein (CRP) to eliminate the potential of confounding effects on the accuracy of 25(OH)D measurements, as it is known, for example, that triglycerides, cholesterol and C-reactive protein (CRP) can exert false negative effects on circulating vitamin D concentrations.[29-32] In addition, these assessments allowed for the evaluation of health status of the study participants.

Test Lab analysis for 25(OH)D

Vitamin D (25(OH)D) was measured using a targeted liquid chromatography with tandem mass spectrometry (LC-MS/MS) method as described elsewhere[33] and using the same sample pretreatment and treatment steps. Calibration solutions or serum samples (50 µL) along with 50 µL of internal standard mixture solution (isotope-labelled- 25(OH)D3-d6) were pipetted into glass vials. This was followed with the addition of a methanol and 0.2 M $ZnSO_4$ mixture (1:1 v/v, 300 µL) for precipitation of serum proteins to facilitate release of 25(OH)D from its binding protein. This was followed with 1 mL of hexane to extract the 25(OH)D. The samples were then vortexed for 10 min and centrifuged at 13,000 rpm for 20 min.

After centrifugation, 650 µL of the hexane layer was taken and dried by evaporation under nitrogen gas at 40 °C. At the end, 200 µL of methanol was added to each dried sample to reconstitute the analytes and 10 µL used for LC–MS/MS analysis. An Agilent 1260 series UHPLC system (Agilent Technologies, Palo Alto, CA) coupled with an AB Sciex QTRAP 4000 mass spectrometer (Sciex Canada, Concord, Canada) with electrospray ionisation (ESI) as the ion source, was used to analyse Vitamin D. A Phenomenex Kinetex C18 column (3.0 mm × 100 mm, 2.6 µm particle size, 100 Å pore size) connected to a Phenomenex Security Guard C18 precolumn (4.0 mm × 3.0 mm), was used to separate 25(OH)D. A gradient elution method was employed. Presented assay was developed to measure 25(OH)D only and the limit of detection (LOD) was 4.69 nM and the calibration range was established as 6.25-400 nM. The multiple reaction monitoring (MRM) was 401.3 > 105.1. The data were analysed using Sciex Analyst 1.6.2.

Reference Lab 1 analysis for 25(OH)D

An Acquity UPLC® BEH phenyl column (1.7 µm, 2.1 x 50 mm) and guard column (Waters Corporation, Milford, MA, USA) were used in the measurements of 25(OH)D by Reference Lab 1. Sample preparation was performed on Tecan Freedom EVO 100 liquid handler (Tecan, Morrisville, NC, USA). First, 100 µL of calibrator, QC or serum sample was mixed with 300 µL of internal standard solution (d6-25(OH)D2 (100 nM) and d6-25(OH)D3 (50 nM) in 50:50 acetonitrile: water). A Waters Acquity UPLC™ (Waters Corporation, Milford, MA, USA) with a triple quadrupole mass detector (Xevo TQD) system in positive electrospray ionisation (ESI) mode was used for the analysis. The dwell time was 40 ms for multiple reaction monitoring (MRM) mode. For optimised measurements, the instrument was set at a capillary voltage of 4.0 kV, desolvation temperature of 500 °C, source temperature of 140 °C, desolvation gas flow at 1200 L/h, cone gas flow at 50 L/h and collision gas flow at 0.20 mL/min. The desolvation gas was provided by a nitrogen generator (PEAK), while the collision gas was argon. Chromatographic separation was achieved by using a BEH phenyl column equipped with guard column at 35 °C with 0.1 % FA in water (mobile phase A) and 100 % acetonitrile (mobile phase B) at a rate of 0.4 mL/min. Initially, the mobile phase composition was 60 % A and 40 % B and mobile phase B was increased to 98 % over 2.8 min and then cycled back to initial conditions 40 % B at 4 min and maintained for 1 min for a total run time 5.0 min. The temperature of the autosampler was set at 10 °C and the injection volume was 37 µL. 25(OH)D2 and 25(OH)D3 fractions were quantitated and total vitamin D levels were reported as their sum. External vitamin D calibrators and quality controls were purchased from Chromsystems (Am Haag, Germany). 25(OH)D3–d6 and 25(OH)D2-d6 were used as the internal standards and were obtained from Medical Isotopes Inc.

Reference Lab 2 analysis for 25(OH)D

Reference Lab 2 utilises the Roche Cobas® e 801 analytical unit for the immunoassay of 25(OH)D, according to procedures described elsewhere.[34] This is a high throughput immuno-chemistry module that performs the immunoassay test for 25(OH)D using the highly innovative and patented Electro-Chemi-Luminescence (ECL) technology. The Elecsys Vitamin D total III assay employs a vitamin D binding protein labelled with a ruthenium complex as a capture protein to bind 25(OH)D3 and 25(OH)D2. Cross reactivity to 24,25(di-OH)D is blocked by a specific monoclonal antibody. The assay system is a fully automated, high throughput immunology analyser for quantitative measuring serum 25(OH)D. For the determination of the 25(OH)D assay repeatability and within-laboratory precision, QC materials for high (63.85 nM) and low (28.0 nM) 25(OH)D according to Clinical Laboratory Standards Institute (CLSI) guideline EP5-A3[35] were used. The linearity of the assay was validated according to CLSI guideline EP6-A.[36] The selection of reference specimen and reference range validation were performed according to CLSI guidelines C28-A3c.[37] The reproducibility and accuracy of the assay system were observed to be 6.3 % and 6.9 % for QC material at lower concentration of 28 nM. Table 1 shows the lab specific reference values used for 25(OH)D, which are different from each

Table 1: Reference values for 25(OH)D from the different analytical laboratories

Laboratory	Sufficient/adequate
Reference Lab 1 (Previous)	30-100 ng/mL (75-250 nM)
Reference Lab 1 (Revised)	20-59.6 ng/mLl (50-149 nM)
Reference Lab 2	30.4-100 ng/mL (76-250 nM)
Test Lab*	12-64 ng/mL (30-160 nM)

**Test Lab measures 25(OH)D3 only, unlike Ref Labs 1 and 2, which measure the sum of 25(OH)D2 and 25(OH)D3.*

other and thus are not interchangeable and cannot be extrapolated to other laboratories.

Statistical analysis

The data were analysed for statistical significance with GraphPad Prism version 9.5.1 (GraphPad Software, Boston, MA, USA), from which the figures were also generated.

Results

General characteristics of study participants

The mean age of the study participants in the initial study was 37.0 ± 1.5 and 33.7 ± 2.2 in the follow up study. The sex distribution among the study population was 75/25 and 55/65 (M/F, %) in the initial and follow up study, respectively. Even though the mean values for the HbA_1c in the initial study (5.43 ± 0.44) and in the follow up study (5.26 ± 0.30) were in the normal (< 5.7 %), 4 participants in the initial study and 1 in the follow up study exhibited HbA_1c values between 5.7 and 6.4 % that were deemed to be prediabetic values. The non-fasted mean values for the study participants for total cholesterol, HDL cholesterol, LDL cholesterol, triglycerides and the ratios of total cholesterol/HDL cholesterol and LDL/HDL were within normal range (data not shown) for the study participants in both the initial and follow up study.

Serum 25(OH)D concentrations

Figure 1 shows the individual values and bar graphs of the serum concentrations of 25(OH)D for the initial study (Figure 1A) and the follow up study (Figure 1B). It can be seen from Figure 1A as well in Table 2 that only 3/40 (7.5 %) values for 25(OH)D measured in the reference laboratory (Reference Lab 1) fell in the lab specific reference values, whereas 23/67 (62.2 %) of the 25(OH)D values obtained in the test laboratory were categorised as being within the lab specific normal reference values in the initial study. Furthermore, 12 (32.4 %) of the individual values for 25(OH)D in the test laboratory were above the upper limit of the reference range of 160 nM). In the initial study, the 25(OH)D value from Reference Lab 1 (45.4 ± 20.8) was approximately 3-fold lower than that obtained from the Test Lab (132.6 ±60.9). This disparity in the 25(OH)D values obtained with the same serum sample from the reference laboratory and the test laboratory led to the follow up study with the inclusion of another

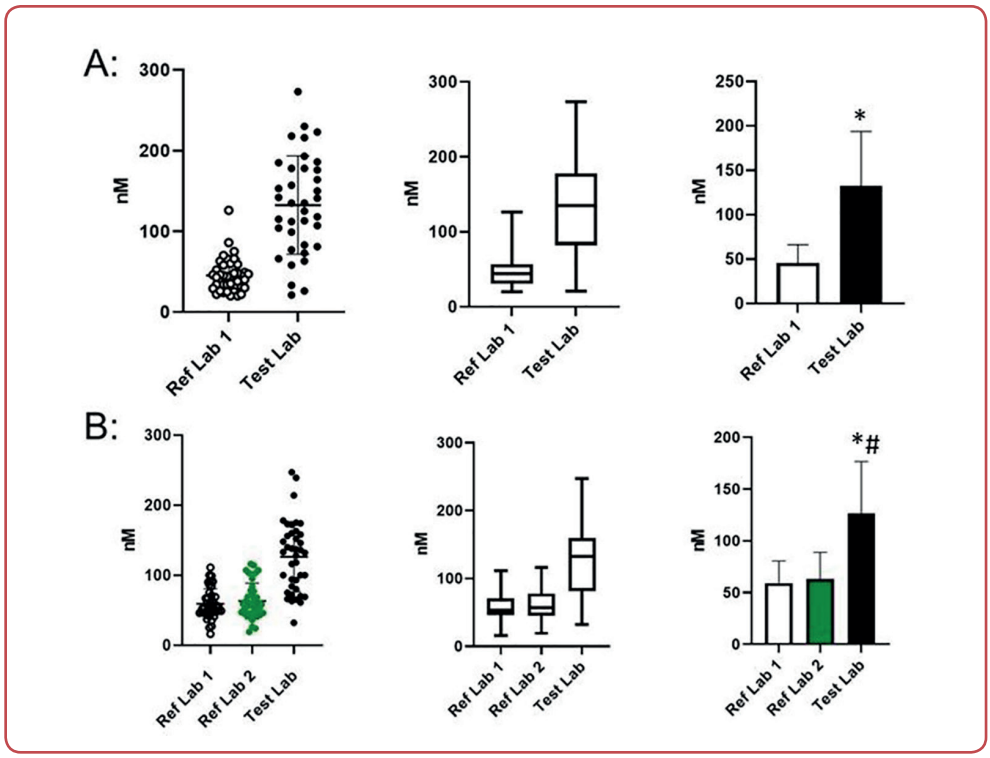

Figure 1: *Serum concentrations of 25(OH)D in the initial and follow up studies*

*Individual values, box plots and bar graphs showing mean ± SE values for serum 25(OH) D in the initial (A) and follow-up (B) studies as measured by the different analytical laboratories. Normal range (reference values) for 25(OH)D for reference laboratory 1 = 75-250 nM (later revised to 50-149 nM in the follow-up study); for reference laboratory 2= 76-250 nM; test laboratory= 30-160 nM. 25(OH)D: 25-hydroxy vitamin D; Ref: reference; lab: laboratory. *p < 0.05 vs Ref Lab 1; #p < 0.05 vs Ref Lab 2.*

reference laboratory (Reference Lab 2) with the capacity to measure serum 25(OH)D. It should be mentioned that prior to sample analysis by the Reference Lab 1 in the follow up study, the reference values were adjusted to 50-149 nM (Table 1). It can be seen from Table 2 and Figure 1B that the individual as well as mean values between Reference Lab 1 and Reference Lab 2 were comparable. However, further analysis of the data revealed that while 23/40 (57.5 %) were within the lab specific normal range for Reference Lab 1, only 11/40 (27.5 %) fell within the normal range for 25(OH) D in Reference Lab 2 (Table 2). While 32/40 (80 %) of the serum values for 25(OH) D were within the reference values in the Test Lab, 8/40 (12 %) were above the upper limit of the reference range (160 nM). Of note the mean values for 25(OH)D obtained from both Reference Lab 1 (59.1 ± 21.6) and Reference Lab 2 (53.9.1 ± 26.3) were approximately 2-fold lower than the mean value for 25(OH)D measured in the Test Lab (126.4 ± 50.1) in the follow up study. Figure 2 depicts the distribution of values of 25(OH)D from Reference Labs 1 and 2 and the Test Lab. It can be seen from Figure 2 A that the majority of the 25(OH)D values obtained from the Reference Lab 1 were narrow whereas those obtained from the Test Lab were more broadly distributed. Similarly, while the distribution pattern for the 25(OH)D levels obtained from Reference Lab 2 in the follow up study were comparable to that seen with Reference Lab 1 (Figure 2B); in contrast the values obtained from the Test Lab in the follow up study were broadly distributed as observed in the initial study.

Further statistical evaluation of the data revealed a correlation of 0.353 with a wide confidence interval, indicating that there is a relatively weak correlation in Test Lab and Reference Lab 1 measurements (Figure 3A). Likewise, a correlation of 0.292 with a wide confidence interval was observed with Test Lab and Reference Lab 2 25(OH)D measurements (Figure 3B). Interestingly, analysis of the measurements of 25(OH)D from both Reference Laboratories revealed a correlation of 0.978 with a narrow confidence interval (Figure 3C). In order to further compare the techniques used in the Test Lab for the measurement of 25(OH) with the existing methods used by the Reference Labs, Bland-Altman analyses were conducted. Bland-Altman analyses of the data demonstrated a bias of -86.7 % with S.D. of bias of 59.2 when comparing the data between the Test Lab and Reference Lab 1, while a bias of -63.3 % with S.D. of bias of 49.2 when comparing the data between the Test Lab and Reference Lab 2 were observed,

Table 2: Proportion of 25-OH vitamin D values within respective laboratory reference values

Laboratory	Reference Lab 1	Reference Lab 2	Test Lab
Initial study	3/40 (7.5 %)	-	23/37[1] (62.2 %)
Follow up study	23/40 (57.5 %)	11/40 (27.5 %)	32/40[2] (80.0 %)

[1] *12 (32.4 %) values at the Test Lab were above upper limit of reference range (160 nM)*
[2] *8 (20.0 %) values at the Test Lab were above upper limit of reference range (160 nM)*

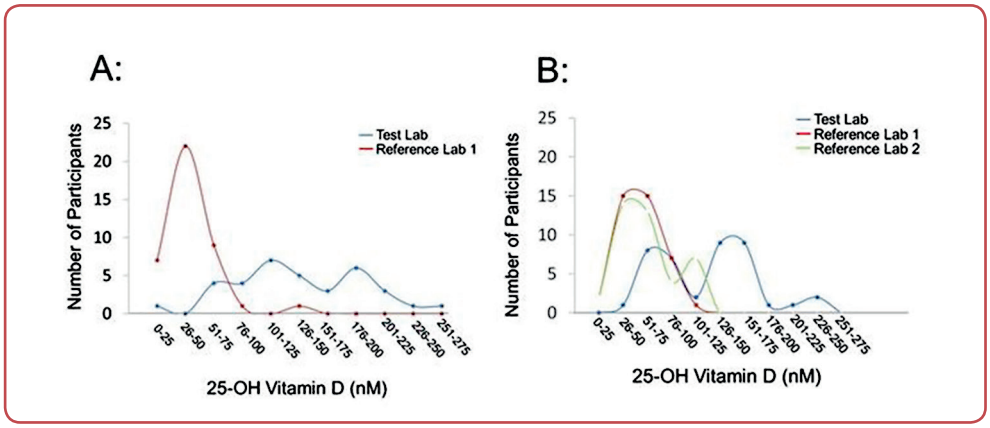

Figure 2: Distribution of 25(OH)D among the different analytical laboratories

Distribution of values for serum 25(OH)D between the test lab and the reference laboratory 1 in the initial study (A) and between the test lab, reference lab 1 and reference lab 2 in the follow-up study (B). 25(OH)D: 25-hydroxy vitamin D; Lab: laboratory;

indicating very poor agreement and higher degree of bias among these assays. In contrast, Bland-Altman analysis of the data between Reference Lab 1 and Reference Lab 2 showed a bias of -4.1 % with SD of bias of 6.4 demonstrating a strong agreement between these assays. It should be noted that no differences in presented test for 25(OH)D with frozen serum was observed (1 freeze thaw cycle, 8 days after collection) or serum kept at 4 °C (8 days) prior to analysis (Figure 4).

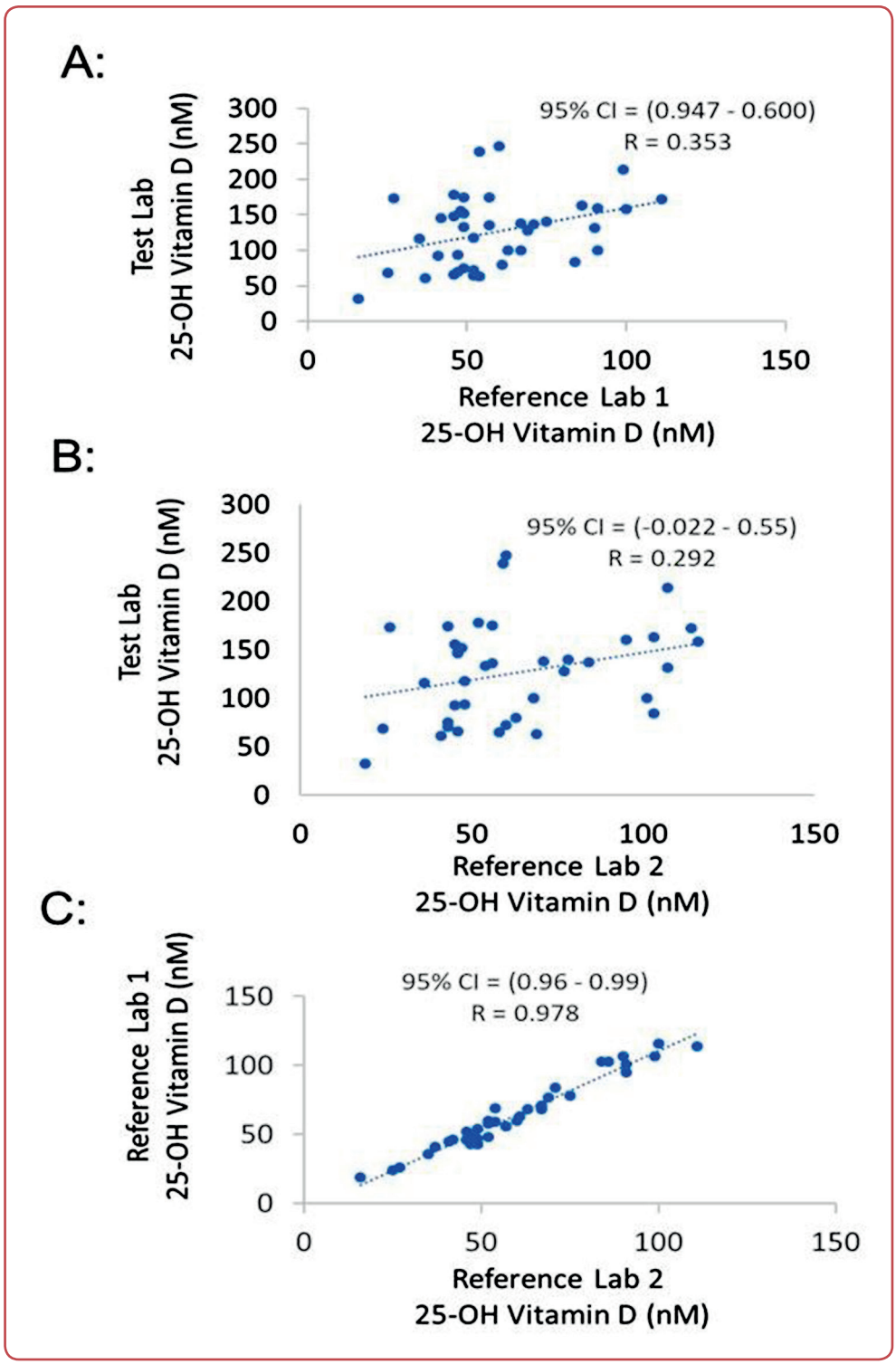

Figure 3: Correlation coefficient values of serum 25(OH)D tested among the different analytical laboratories

Correlation and confidence intervals for serum 25(OH)D values between the test lab and reference laboratory 1 (A), test lab and reference lab 2 (B) and reference lab 1 and reference lab 2 (C). 25(OH)D: 25-hydroxy vitamin D; R: correlation coefficient; CI: confidence interval.

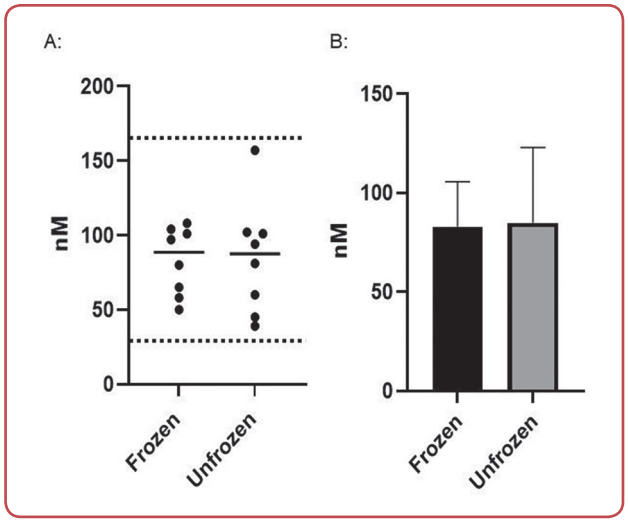

Figure 4: Effect of freeze-thaw and storage of serum on 25(OH) D analysis

Individual values (A) and bar graphs (B) showing mean ± SE values for serum 25(OH) D in the frozen and unfrozen serum samples as measured by the test lab. Normal range as indicated in dashed lines, 30-160 nM. 25(OH)D:25-hydroxy vitamin D.

Discussion

In view of the high global occurrence of vitamin D deficiency and its association with diverse diseases, vitamin D testing has markedly increased worldwide.[8-17] It is crucial for all laboratories to be aware of the performance and limitations of their 25(OH)D assays.[38] A variety of methods including immunoassays and chromatographic techniques have been employed to measure 25(OH)D concentrations in the serum with ongoing refinements designed to improve accuracy, reliability and sensitivity.[39-48] Indeed, by applying rigorous quality control strategies in LC-MS/MS determination of 25(OH)D, laboratories can achieve greater analytical performance and deliver more accurate clinical results..[49] Biosensors have recently been developed as promising options for routine vitamin D analysis.[19] Furthermore, the analysis of a small amount of urine using the nanoluc-based vitamin D receptor assay or ELISA may be useful as a proxy for predicting the serum 25(OH)D levels.[50, 51]

The present study was undertaken to adapt and validate a new method for determining 25(OH) D concentrations in an ostensibly healthy population. However, the study revealed an inconsistency in the data obtained from our Test Lab as compared to two gold standard reference analytical laboratories. The goal of the Vitamin D Standardisation Certification Program is for accuracy of 25(OH)D concentration to be within 5 % of the Standard Reference Material (SRM) values with a bias of ± 5-10 % as satisfactory.[52] Accordingly, further analysis of data through Bland-Altman analysis revealed that there is a trend for more variation as the concentration increases, which is proportional bias. This bias (when comparing Test Lab values to Reference Labs 1 and 2) was larger than a typical standard deviation, suggesting that very large biases continue to be observed. In other words, the techniques used by the Test Lab to measure 25(OH)D do not agree equally throughout the range of measurements as obtained by both Reference Labs and thus indicating systematic differences as opposed to random errors. Despite the prominent international efforts to standardise serum 25(OH)D measurements, inconsistencies in the measurements still exist. The present study has demonstrated that 25(OH)D assay by independent laboratories produce varying results from the same participant specimen.

It should be mentioned that during the interim period between completion of the initial study and start of the follow up study, Reference Lab 1 revised their normal reference values for 25(OH) D, with a narrower range and lower cut off points, ie from 75-250 nM to 50-149 nM. In contrast, the optimal range for 25(OH)D at Reference Lab 2 (76-250 nM) and the Test Lab (30-160 nM) did not change. In the follow up study, with the amended reference range for 25(OH)D used Reference Lab 1, 42 % of the study cohort was deemed to be either below normal range (< 50 nM) or deficient (< 30 mM) for 25(OH)D levels, which is similar to the 2011-2012 NHANES values.[53] In the follow up study, while according to the Test Lab 80 % of the study participants exhibited values for 25(OH)D to be in their normal range, 8 of the 40 had higher values that were over the normal range.

Manson et al[54] have defined normal reference values for 25(OH)D as 40-80 ng/mL (100-200 mM), while Sonderman et al[55] have defined sufficient/adequate levels as ≥ 20 ng/mL (50 nM). Presented reference values (12-64 ng/mL (30-160 nM) were largely derived from these important studies as well as from the Institute of Medicine, Food and Nutrition Board.[56] Although other bodies have also declared reference values for 25(OH)D that are based on bone and overall health in healthy individuals, optimal and deficiency 25(OH) status are yet to be established as there are differ-

ences with respect to geographic/ethnic/age and sex considerations. In addition, reference values were measured using older technologies. Thus, large study to establish a reference range in the normal population is warranted.

While the use of SRM is crucial in obtaining reliable and accurate 25(OH)D values, some procedures including sample handling, preparation and extraction may also influence outcomes that could contribute to some of the inconsistencies in 25(OH)D levels observed among different laboratories. For instance, although the exact methodologies employed for 25(OH)D by the reference laboratories is propriety information, it can be suggested that the differences in sample preparation among the analytical laboratories may explain the discrepancy in the values for 25(OH)D, particularly between the Test Lab and the Reference Laboratories. In this regard, Reference Lab 1 employs an automated sample preparation, while the system at the Test Lab is not a fully automated system, but a combination of automated plus manual system with respect to use of multichannel pipette, centrifugation conducted in a 96 well plate. While complete information regarding sample preparation/treatment particularly as it relates to separating vitamin D from its binding protein, is a propriety process, this could potentially be a source of the reported differences. Pre-analytical stability of 25(OH)D may be a source of the disparity in the values obtained from the Reference Laboratories and the Test Lab. Indeed, the literature has consistently reported that instability during storage or transport constitutes a significant source of variability and potential inaccuracy in 25(OH)D assays.[57]

Evidence indicates that vitamin D–binding protein exhibits substantial stability at ambient (room) temperature, including in unprocessed blood specimens. Furthermore, delays of several hours prior to analysis, whether in processed or unprocessed samples, have minimal impact on measured concentrations. Even samples received unfrozen or inadvertently left at room temperature remain largely suitable for analysis, as observed reductions after three days under typical laboratory conditions are within the analytical inter-assay variability. Consequently, freezing serum prior to transport appears unnecessary and whole blood may even represent the preferred specimen type for transportation lasting up to three days. Additionally, serum stored at 4 °C remains stable for at least seven days and tolerates up to four freeze–thaw cycles without significant degradation. In fact, 25(OH)D is highly stable at room temperature.[39, 40] Thus, degradation or loss of integrity of the samples can be excluded in view of the above as well as our own data (Figure 4). In addition, age (aging) and sex (hormonal differences) did not have any influence on the 25(OH)D concentrations (data not shown).

It should be pointed out that in the study by Colak et al,[58] 25(OH)D was assessed in samples that were 8-12 h fasted. However, a recent study[59] has reported there is not much difference with fasting and that studies with small sample sizes before/after supplement use, observed only a difference of 8 nmol/L.[59] Of particular importance is awareness of the cross-reactivity of 3-epi-25 (OH)D, which interferes with the assay; indeed, most LC-MS/MS techniques are unable to differentiate between C3 epimer, 3-epi-25 (OH) D and 25(OH)D, contributing to a overestimation of results.[60] Under certain conditions, such elevations may artifactually normalise serum vitamin D concentrations in deficient individuals or, in more pronounced cases, result in apparent supraphysiological levels approaching toxicity.[61-64] It should be noted that many automated immunoassay systems incorporate biotinylated antibodies and streptavidin coated magnetic beads for amplified signals, precision, high sensitivity and specificity.[65, 66] However, the presence of high biotin in analytical samples cause interference,[67, 68] which can be eliminated by employing biotin neutralisation techniques.[65]

From the aforementioned, the quality and source of materials that the different analytical laboratories use to standardise their assays may also differ and contribute to the observed discrepancies. Indeed, any differences in the internal reference material for master curve standards could result in variable data between the laboratories. Therefore, implementing rigorous quality control standards for the LC–MS/MS quantification of 25(OH) D, characterised by high analytical performance, will enable more precise and reliable vitamin D measurements for clinical applications.[69] In this regard, in view of the analytical concerns raised regarding method comparisons and the accuracy of the different assays employed to measure vitamin D status, the National Institute of Standards and Technology along with the office of Dietary Supplements of the National Institutes of Health, brought forward the first SRM® in 2009 for use in assessing vitamin D metabolites.[70] Sub-

sequently, there are now five SRMs and three calibration SRMs. It may be argued that presented assay system has not employed SRMs for method robustness and instrument variability. In this regard, the validation of presented assay as well as method robustness, instrument variability and quality control were conducted by spiking serum samples with a known concentration of the vitamin (isotope-labelled 25-OH-Vitamin D3-d6) to confirm recovery, linearity and precision with respect to inter- and intra- daily reproducibility. It is pointed out that we performed a recovery test instead of matrix effect and the recoveries at low, mid and high levels were within accepted 80-120 %.

It should be mentioned that in the initial study, 4/39 study volunteers and 1/40 study volunteers in the follow-up study exhibited values for CRP above the normal reference value of 5 mg/L (data not shown). In this small sample cohort, the higher CRP level did not correspond to lower vitamin D levels. While some studies have demonstrated an association of low 25(OH)D with CRP,[71-75] there is some disagreement if optimal vitamin D levels are causally linked to diminished inflammation or if inflammation itself reduces 25(OH)D concentrations.[76] In addition, 2/40 in both the initial and follow-up study exhibited HbA_1c values between 6.1 and 6.4 % that placed them in a pre-diabetic stage[77] (data not shown). While vitamin D deficiency has been proposed as a risk for the development of type 2 diabetes,[78] low 25(OH) concentrations in these study volunteers were not observed (data not shown). From the aforementioned, a longitudinal follow-up and mass screenings for 25(OH)D status in different populations will help ascertain the reasons for the discrepancies in the measurements of vitamin D for which a validated assay system is crucial[79] including methodological comparability.[80] Since circulating 25(OH)D is mostly protein bound, it has been proposed that the free (non-protein bound) serum 25(OH)D could be a more accurate determinant of vitamin D status than total 25(OH)D.[81,82] In this context, establishing target values for free 25(OH)D in SRMs would enable the validation of novel analytical methods and facilitate the harmonisation of measurement procedures.[81]

In spite of the existence of substantial data, consensus on the optimal as well as deficiency values for 25(OH)D remains to be attained. While two thresholds of 20 ng/mL or 30 ng/mL have been recognised, the higher cut-point, which was initially recommended by the Endocrine Society, is now no longer supported (*https://www.endocrine.org/*), thus 20 ng/mL is generally used. It should be mentioned that in North America, a serum concentration of 40 nM 25(OH)D is considered adequate while a concentration of < 30 nM 25(OH)D is deemed as a deficiency; 50 nM is considered sufficient and concentrations > 125 nM would be considered abnormal and at risk for adverse effects.[83] Notwithstanding the recognised variability in diagnostic thresholds for vitamin D insufficiency and deficiency, formal guidelines regarding the optimal timing and frequency of screening remain absent.[84, 85] While the analysis and interpretation of 25(OH)D data is challenging, the metabolism of vitamin D is complex, which is further complicated with the fact that genetic polymorphisms can influence serum vitamin D concentrations.[86, 87] With respect to the complex nature of the determination of vitamin D status, because of the interindividual variability in response to vitamin D supplementation in deficiency, It has been proposed that the concurrent measurement of 25(OH)D, $24,25(OH)_2D$ and additional metabolites provides a more comprehensive assessment of an individual's vitamin D state.[88]

Conclusion

In contrast to Reference Laboratories 1 and 2, which quantify the combined concentrations of $25(OH)D_2$ and $25(OH)D_3$, the Test Lab measures $25(OH)D_3$ exclusively. Despite this very important distinction, the present study has illustrated the inconsistency of results from different laboratories and that a very large bias was observed when comparing Test Lab 25(OH)D values to those from both Reference Laboratories. From a diagnostic perspective, these differences are substantial enough to influence the classification of participants as having sufficient or deficient 25(OH)D levels. These outcomes highlight the importance of standardisation of vitamin D analyses, limitation of errors as well as the need for harmonisation of results that will accelerate improvements in methods and accurate, reproducible measurements for vitamin D with diagnostic value. It is possible that some laboratories are not yet using the National Institute of Standards and Technology (NIST) SRMs and as

such interpretation at the more individual level remains inconsistent with the goals of Vitamin D Standardisation Program and Vitamin D Standardisation Certification Program, thus more work remains to be done.

Ethics

This study was approved by the University of Manitoba Biomedical Research Ethics Board (Ethics File Number: HS20924 (B2017:090)), dated 10 November 2017; as well as by the St. Boniface Hospital Research Review Committee (File Number: RRC/2017/1682), dated 16 November 2017. All volunteers enrolled into the study consented prior to participation.

Acknowledgement

Infrastructural support was provided by the St Boniface Hospital Albrechtsen Research Centre.

Conflicts of interest

The authors declare that there is no conflict of interest.

Funding

The work reported here was supported by funding from the National Research Council of Canada Industrial Research Assistance Program (NRC-IRAP).

Data access

The data that support the findings of this study are available from the corresponding author upon reasonable individual request.

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