

Quantification of Serum 25-Hydroxyvitamin D₂ and D₃ Using HPLC–Tandem Mass Spectrometry and Examination of Reference Intervals for Diagnosis of Vitamin D Deficiency

Amy K. Saenger, PhD, Thomas J. Laha, Deborah E. Bremner, and Sayed M.H. Sadrzadeh, PhD

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Abstract

Serum levels of 25-hydroxyvitamin D are important in establishing true vitamin D levels in humans. The purposes of this study were to develop a sensitive, specific liquid chromatography–tandem mass spectrometry (LC-MS/MS) method for detection of 25-hydroxyvitamin D₂ and D₃ and establish reference intervals for these analytes. Chromatographic separation of 25(OH)D₂ and 25(OH)D₃ was achieved after adding deuterated Δ^9 -tetrahydrocannabinol (Δ^9 -THC-D₃) and organic extraction. The 3 ions were ionized using positive electrospray ionization and detected in the multiple-reaction monitoring mode using mass (m)/charge (z) transitions of 318.15 > 196.20 (Δ^9 -THC-D₃), 401.15 > 365.2 [25(OH)D₃], and 413.15 > 355.20 [25(OH)D₂]. Reference interval study results were compared with current 25(OH)D recommendations.

Elution of 25(OH)D₂, 25(OH)D₃, and Δ^9 -THC-D₃ was achieved after 3.0 minutes (total run time, 6.0 minutes). Within- and between-run coefficients of variation were less than 11%. Deming regression of radioimmunoassay and LC-MS/MS methods for total 25(OH)D levels yielded a slope of 0.97 (95% confidence interval, 0.88–1.05) and y-intercept of -1.74 ng/mL. Reference intervals were less than recommended levels (D₂, 0.0–12.1; D₃, 5.5–41.4; total vitamin D, 6.0–43.5 ng/mL [0–30, 14–103, 15–109 nmol/L, respectively]) with no statistically significant differences in race, age, or sex. This LC-MS/MS method provides a rapid, accurate, sensitive, and cost-effective alternative to other methods for detection of 25(OH)D₂ and 25(OH)D₃ at nanomolar concentrations.

Hypovitaminosis D is a relatively common problem traditionally manifesting in the elderly population and in people with severe liver or kidney disease.^{1,2} Vitamin D deficiency has increasingly manifested in the general population due to lack of sun exposure, poor nutritional status, and increased use of sunscreens.^{3,4} Inadequate levels of vitamin D can lead to increased risk of cancer, diabetes mellitus, chronic pain, and hypertension.^{5–10} Vitamin D₂ (ergocalciferol) and D₃ (cholecalciferol) may come from exogenous sources, but only vitamin D₃ is produced in the skin from 7-dehydrocholesterol upon exposure to sunlight. Vitamin D₂ and D₃ are metabolized to 25-hydroxyvitamin D [25(OH)D₂ and 25(OH)D₃] in the liver and subsequently converted to the biologically active 1,25-dihydroxyvitamin D form in the kidneys. Circulating levels of 25-hydroxyvitamin D have been demonstrated to be the most precise marker of a patient's vitamin D status.

Although vitamin D₂ is generally present in significantly smaller quantities than vitamin D₃, measurement of 25(OH)D₂ and 25(OH)D₃ has an important role in assessing clinical nutritional status. Patients with severe vitamin D deficiency receive therapeutic doses consisting solely of vitamin D₂ (up to 50,000 IU), thus quantification of 25(OH)D₂ levels is extremely important to monitor treatment effectiveness.

Several methods, including high-performance liquid chromatography (HPLC), chemiluminescence, and radioimmunoassay (RIA) have been developed for measurement of total 25(OH)D levels. Although HPLC techniques with UV detection are capable of determining 25(OH)D₂ and 25(OH)D₃ levels simultaneously, there are significant drawbacks. Most HPLC methods require large sample volumes (0.5–2 mL) and time-consuming procedures before quantification.¹¹ Suboptimal cross-reactivity of the antibody with 25(OH)D₂ causes underrecovery

of 25(OH)D₂ in chemiluminescent immunoassays.¹² Although RIAs eliminate the need for large sample volumes, they cannot distinguish between 25(OH)D₂ and 25(OH)D₃. In addition, RIA methods involve cumbersome waste disposal and have decreased precision at low concentrations.

Liquid chromatography–tandem mass spectrometry (LC-MS/MS) is a highly sensitive technique and is considered the “gold standard” for detection and quantification of numerous analytes. Few LC-MS/MS methods have been described for quantitation of 25(OH)D₂ and 25(OH)D₃; however, all require derivatization and/or expensive deuterated internal standards. For example, the 25(OH)D LC-MS/MS method described by Higashi et al¹³ had no sample pretreatment requirements but required the use of derivitization methods with a Cookson-type reagent. Tsugawa et al¹⁴ introduced a liquid chromatography–atmospheric pressure chemical ionization mass spectrometry method using deuterated 25(OH)D₃ as the internal standard. The shortcomings of the latter method were complex synthesis of the internal standard and relatively long chromatographic times (11 minutes). Vogeser et al¹⁵ modified the extraction procedure by using solid-phase extraction to obtain further purification before quantification of 25(OH)D₂ and 25(OH)D₃. Recently, Maunsell et al¹⁶ used isotope-dilution LC-MS/MS for detection of 25(OH)D₂ and 25(OH)D₃ using a deuterated vitamin D₃ internal standard with longer analysis times (8 minutes).

This article describes a sensitive LC-MS/MS method for the detection and quantification of 25(OH)D₂ and 25(OH)D₃ using a novel internal standard, deuterated Δ⁹-tetrahydrocannabinol (THC)-D₃ with shorter assay analysis times. Reference interval data were obtained for 25(OH)D₂ and 25(OH)D₃ and are discussed in relation to the Northwest population and recent recommendations for increasing desirable levels of total 25(OH)D.

Materials and Methods

Chemicals and Reagents

Absolute ethyl alcohol was purchased from Aaper Alcohol and Chemical (Shelbyville, KY), and ammonium acetate was obtained from ICN Biomedicals (Costa Mesa, CA). HPLC-grade methanol and *n*-heptane were acquired from J.T. Baker (Phillipsburg, NJ) and Fischer Scientific (Fairlawn, NJ), respectively. 25(OH)D₂ and 25(OH)D₃ were purchased from Sigma-Aldrich (St Louis, MO). These 2 lyophilized powders were diluted with ethanol and used to make working standards (concentrations of each analyte: 1, 30, and 100 ng/mL). Control samples were prepared from pooled serum specimens that were assayed and spiked with vitamin D

standards with optimal control values for 25(OH)D₂ and 25(OH)D₃ less than 15 ng/mL (low) and 20 to 40 ng/mL (high). The internal standard, Δ⁹-THC-D₃ (Cerilliant, Round Rock, TX), was diluted with methanol to a final concentration of 50 ng/mL.

Sample Preparation

For each patient serum specimen and control sample, 200 μL of sample was placed in a glass 13 × 100-mm disposable tube followed by 200 μL of internal standard. The samples were vortex-mixed, extracted with 1 mL of *n*-heptane, and centrifuged for 4 minutes at 3,000 rpm. The organic layer was removed, evaporated under nitrogen, and reconstituted in 100 μL of ethyl alcohol. After brief vortex mixing, the samples were transferred to labeled auto-sampler vials. Control samples were extracted with patient samples during every run. The standards were assayed unextracted, with 100 μL of standard mixed with 200 μL of internal standard. This solution was vortex-mixed and dried under nitrogen. Standards were reconstituted in ethyl alcohol, and the solutions were transferred into auto-sampler vials. Then, 20 μL of each standard, control sample, or patient specimen was injected into the LC-MS/MS system.

LC-MS/MS Instrumentation and Analysis

The LC-MS/MS system consisted of a Waters Alliance 2795 HPLC interfaced to a Waters Micromass Quattro tandem quadrupole mass spectrometer (Waters, Milford, MA). Chromatographic separation of 25(OH)D₂, 25(OH)D₃, and Δ⁹-THC-D₃ was achieved with a Waters XTerra analytical column (50 × 2.1 mm internal diameter; 3.5-μm particle size; Waters) maintained at a temperature of 35°C. An isocratic mobile phase was used and consisted of 100% methanol with 2 mmol/L of ammonium acetate and 0.1% formic acid. The flow rate was 100 μL/min from 0.0 to 4.0 minutes, at which point all compounds of interest were eluted. The flow then was increased to 1 mL/min and diverted to waste as a wash step. At 5.9 minutes, the flow decreased back to 100 μL/min. The total chromatographic run time for each sample was 6.0 minutes, and typical elution times for 25(OH)D₂, 25(OH)D₃, and Δ⁹-THC-D₃ were 2.90, 2.89, and 2.93 minutes, respectively. The mass detector acquisition and tune settings are shown in **Table 1** and **Table 2**. Electrospray ionization was performed in the positive mode.

Δ⁹-THC-D₃, 25(OH)D₃, and 25(OH)D₂ were monitored in the multiple-reaction monitoring mode using the following transitions: 318.15 > 196.20 for Δ⁹-THC-D₃, 401.15 > 365.25 for 25(OH)D₃, and 413.15 > 355.20 for 25(OH)D₂. Quantification was performed using MassLynx 4.0 software (Waters) using integrated peak area ratios of 25(OH)D₂/Δ⁹-THC-D₃ or 25(OH)D₃/Δ⁹-THC-D₃.

Table 1
Liquid Chromatography–Tandem Mass Spectrometry Instrument Conditions: MRM Acquisition Settings

	MRM <i>m/z</i> Pairs	Dwell (s)	Cone (V)	Collision (eV)	Delay (s)
25(OH)D ₂	413.15 > 355.20	0.2	15.0	10.0	0.03
25(OH)D ₃	401.15 > 365.15	0.2	15.0	12.0	0.03
Δ ⁹ -THC-D ₃	318.15 > 196.20	0.2	35.0	20.0	0.03

m, mass; MRM, multiple-reaction monitoring; 25(OH)D₂, 25-hydroxyvitamin D₂; 25(OH)D₃, 25-hydroxyvitamin D₃; Δ⁹-THC-D₃, deuterated Δ⁹-tetrahydrocannabinol; *z*, charge.

Method Validation

The precision, linearity, and limit of detection were evaluated to assess the performance characteristics of the assay. Interassay precision was assessed for 25(OH)D₂ and 25(OH)D₃ using a minimum of 20 low and high control samples for each analyte extracted in multiple runs per day. Intra-assay precision used the same control samples with a minimum of 20 times within a run. Assay linearity was determined by serially diluting spiked serum samples with saline and comparing results with theoretical values. Twenty replicate measurements of the saline blank were used to determine the lower limit of detection, defined as the mean of the negative sample plus 2 SD. The biological limit of detection was established by using the lower limit of detection plus 2 SD obtained from a saline-diluted serum pool to achieve 3 low concentrations for 25(OH)D₂ and 25(OH)D₃ using 20 replicate experiments. Functional sensitivities were calculated based on the lowest analyte concentrations of the diluted serum pools with interassay coefficients of variation (CVs) of less than 20%. Analytical recovery was determined by adding the vitamin D analytes to serum at 3 concentrations (5.0, 15.0, and 100.0 ng/mL) and analyzing the specimens.

Method Comparison and Reference Intervals

The LC-MS/MS method was compared with the DiaSorin RIA (Stillwater, MN) by using aliquots from 57 patient serum samples. Deming regression was used for statistical comparison of the methods. Serum samples from 110 healthy individuals also were assayed to assess reference intervals for 25(OH)D₂ and 25(OH)D₃ and to verify the reference interval used with the RIA method. Approval for these studies was obtained from the University of Washington Institutional Review Board (Seattle). Volunteers answered a questionnaire about race, age, sex, medications, and exogenous vitamin D intake. Reference interval data were analyzed according to the National Committee for Clinical Laboratory Standards guideline for determining reference values.¹⁷ Data points were placed in decreasing order and evaluated to determine outliers. The central 95% of the data were taken as the reference interval for each analyte.

Serum calcium and plasma parathyroid hormone (PTH) levels were measured for comparison with total 25(OH)D levels

Table 2
Liquid Chromatography–Tandem Mass Spectrometry Instrument Conditions: Tune Settings

Parameter*	Setting
LM1	15.0
HM1	15.0
LM2	14.0
HM2	14.0
Source (C)	130
Desolvation (C)	400
Capillary (kV)	4.00

C, Celsius; HM, high mass; LM, low mass.

* LM and HM refer to the resolution.

and with abnormal levels used as exclusion criteria for the reference interval study. Serum calcium and plasma PTH levels were measured on the Beckman LX20 (Beckman Coulter, Fullerton, CA) and Roche Elecsys 1010 (Roche Diagnostics, Mannheim, Germany), respectively. Plasma and serum specimens were separated and stored at -70°C until analysis.

Results

Examples of extracted ion chromatograms for 25(OH)D₂, 25(OH)D₃, and Δ⁹-THC-D₃ are shown in **Figure 1**. Ion suppression was tested by HPLC analysis of an extracted serum specimen along with postcolumn infusion of 25(OH)D₂, 25(OH)D₃, and Δ⁹-THC-D₃. Minimal matrix ion suppression was compensated for by use of a coeluting internal standard when analyte/internal standard ratios were used for quantitation.

The performance characteristics of the LC-MS/MS method are given in **Table 3**. Interassay CVs were 11.4% to 11.5% and 7.7% to 9.8% and intra-assay CVs were 6.9% to 8.8% and 6.0% to 8.0% for 25(OH)D₂ and 25(OH)D₃, respectively. Absolute recovery of vitamin D₂ from spiked serum samples ranged from 93% to 106%, whereas vitamin D₃ recovery was 86% to 92%. The LC-MS/MS method was linear over the working range from 1.0 to 100.0 ng/mL ($r > 0.999$) for 25(OH)D₂ and 25(OH)D₃ (data not shown). The lower limits of detection were 0.09 ng/mL and 0.06 ng/mL for 25(OH)D₂ and 25(OH)D₃, respectively, and the biological

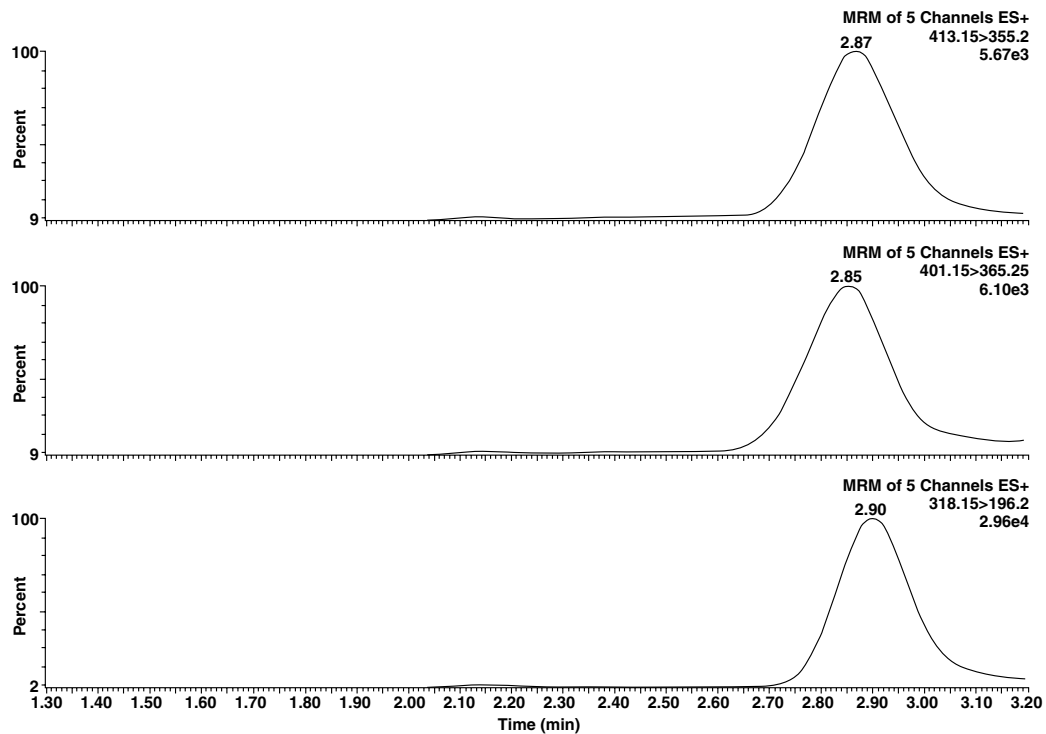


Figure 1 Liquid chromatography–tandem mass spectrometry (LC-MS/MS) ion chromatograms of an extracted serum specimen. The LC-MS/MS chromatogram illustrates analyte concentrations of 29.0 ng/mL for 25-hydroxyvitamin D₂ and 24.4 ng/mL for 25-hydroxyvitamin D₃. The concentration of the internal standard, deuterated Δ⁹-tetrahydrocannabinol, was 50 ng/mL. Numbers at the tops of the peaks represent the time of the peak maximum. ES, electrospray; MRM, multiple-reaction monitoring.

limits of detection also were well below the linear limits of the assay (D₂, 0.3 ng/mL; D₃, 0.1 ng/mL). Functional sensitivities were 0.7 and 0.1 ng/mL for 25(OH)D₂ and 25(OH)D₃. No carryover was evident on the LC-MS/MS instrument at concentrations up to 200 ng/mL. Possible interference by hemolysis, ictericia, or lipemia was analyzed with the LC-MS/MS assay and found to have no interference with measurement of 25(OH)D. Both hydroxylated forms of vitamin D were stable during the course of extraction, during quantification, and during several freeze-thaw cycles. 25(OH)D has been shown to be stable up to 2 years in frozen serum and 72 hours in uncentrifuged blood at 24°C.^{18,19}

To assess correlation of the LC-MS/MS method with an RIA, 57 patient serum specimens were analyzed by both methods. As shown in **Figure 2**, Deming regression analysis showed that the methods were comparable and yielded a slope of 0.97 (95% confidence interval, 0.88-1.05) and y-intercept of -1.74 ng/mL. It should be noted that only total vitamin D levels were evaluated owing to the cross-reactivity properties of the RIA.

To establish our reference range, serum samples from 116 healthy volunteers (age range, 22-65 years) were analyzed for calcium, PTH, 25(OH)D₂, and 25(OH)D₃ levels. The serum calcium levels were normal in all (mean ± SD, 9.8 ± 0.4

Table 3
Performance Characteristics of the Liquid Chromatography–Tandem Mass Spectrometry Assay for 25(OH)D₂ and 25(OH)D₃

	25(OH)D ₂	25(OH)D ₃
Intra-assay CV (%)	6.9-8.8	6.0-8.0
Interassay CV (%)	11.4-11.5	7.7-9.8
Lower limit of detection (ng/mL)	0.09	0.06
Biologic limit of detection (ng/mL)	0.3	0.1
Functional sensitivity (ng/mL)	0.7	0.1
Recovery (%)	93-106	86-92

CV, coefficient of variation; 25(OH)D₂, 25-hydroxyvitamin D₂; 25(OH)D₃, 25-hydroxyvitamin D₃.

mg/dL [2.45 ± 0.10 mmol/L]), but samples from 6 volunteers were excluded from the study owing to an elevated PTH level. Their mean serum PTH level was 39 ± 12 pg/mL (4.1 ± 1.3 pmol/L; range, 13-74 pg/mL [1.4-7.8 pmol/L]).

The distribution of results for 25(OH)D₂ and 25(OH)D₃ was nongaussian **Figure 3**. The calculated reference intervals based on the central 95% of the data were 0.0 to 12.1 ng/mL (0-30 nmol/L) for 25(OH)D₂, 5.5 to 41.4 ng/mL (14-103 nmol/L) for 25(OH)D₃, and 6.0 to 43.5 ng/mL (15-109 nmol/L) for total vitamin D. The median, 10th, and 90th percentiles of

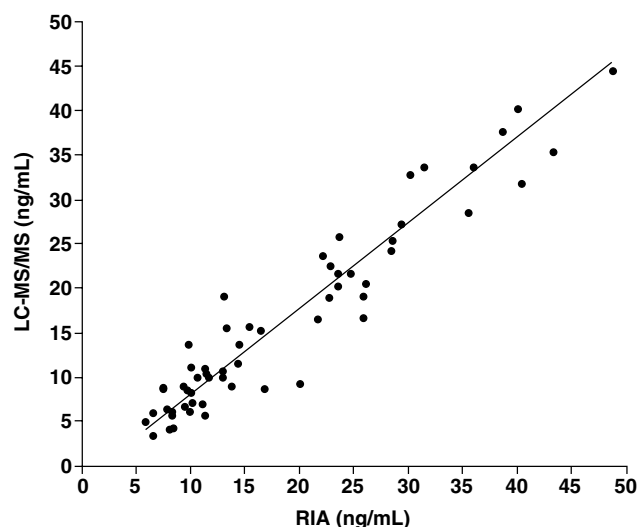


Figure 2 Deming regression comparing total 25-hydroxyvitamin D levels obtained by liquid chromatography–tandem mass spectrometry (LC-MS/MS) and radioimmunoassay (RIA) methods using 57 patient serum samples. Analysis showed that both methods were comparable and yielded a slope of 0.97 (95% confidence interval, 0.88-1.05) and a y-intercept of -1.74 ng/mL; $r = 0.96$.

25(OH)D₂, 25(OH)D₃, and total 25(OH)D for all subjects were 1.8, 0.0, and 15.3 ng/mL (4, 0, and 38 nmol/L); 13.4, 4.2, and 37.1 ng/mL (33, 10, and 93 nmol/L); and 16.1, 6.1, and 33.7 ng/mL (40, 15, and 84 nmol/L), respectively. We observed no statistically significant differences as a result of sex, race, or age. Volunteers who took daily vitamin D supplements had significantly higher total 25(OH)D values than those

who did not (23.7 vs 14.1 ng/mL [59 vs 35 nmol/L]). The current decision intervals used at our institution are 15.1 to 50 ng/mL (normal; 37-125 nmol/L), 8.0 to 15 ng/mL (deficient; 20-37 nmol/L) and less than 8 ng/mL (severely deficient; <20 nmol/L). Therefore, based on our existing reference range, 51% of volunteers with “normal” results in our study had vitamin D deficiency (<15.0 ng/mL [<37 nmol/L]).

Discussion

A major problem in determination of a patient’s true vitamin D status can be attributed to the hydrophobic, lipophilic characteristics of vitamin D. The strong binding properties of vitamin D binding protein and the similar structural features of vitamin D₂ and D₃ also contribute to complexities in vitamin D measurement. Commonly used RIA and chemiluminescence methods claim 75% and 100% cross-reactivity, respectively, between 25(OH)D₂ and 25(OH)D₃.^{12,20} Difficulties are encountered in patients with severe vitamin D deficiency due to compromised metabolism of vitamin D or from a combination of lack of sun exposure and inadequate dietary intake. These patients typically receive large doses of vitamin D₂ and, thus, require accurate monitoring of 25(OH)D₂ levels to establish proper dosing intervals and treatment efficacy. We have developed a precise assay that can measure both forms of 25(OH)D accurately.

Internal standards typically are structurally analogous to the analytes of interest in chromatographic methods. However, in most cases, finding an appropriate internal standard can be cumbersome and expensive. For example, stable isotopes of vitamin D require lengthy synthesis, and, at the

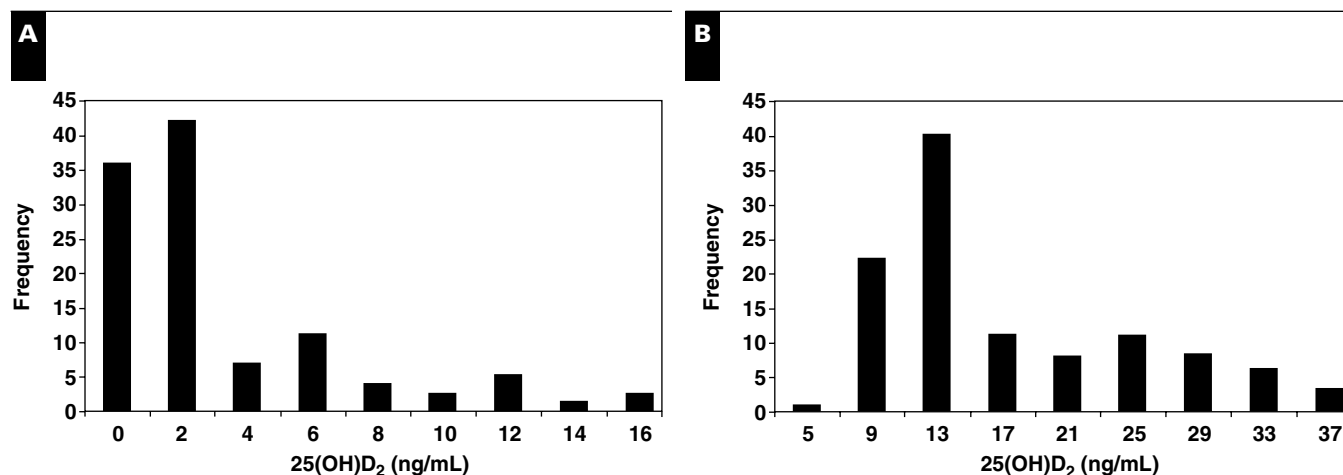


Figure 3 Reference interval data and distribution for 25-hydroxyvitamin D₂ [25(OH)D₂] (A) and 25-hydroxyvitamin D₃ [25(OH)D₃] (B). Results are from 80 healthy volunteers who provided serum samples for the study. No significant differences were observed in sex, age, or race. Distribution shows the nongaussian trend in the data, and median values for all subjects were as follows: 25(OH)D₂, 1.8 ng/mL (4 nmol/L); 25(OH)D₃, 13.4 ng/mL (33 nmol/L); and total 25(OH)D, 16.1 ng/mL (40 nmol/L).

time of initial method development, there were no readily commercially available deuterated vitamin D compounds. After extensive research, we decided to use Δ^9 -THC- D_3 , a compound that shares similar chemical and structural properties to vitamin D, as a novel and cost-effective alternative. Internal standards that are not structurally identical to the compound of interest are susceptible to the consequences of ion suppression, and a variety of methods have been described to counteract its effects.²¹ LC-MS/MS methods frequently encounter issues with ion suppression; thus, it is critical to minimize or compensate for ion suppression if found to be present.²¹ Significant ion suppression was observed during our initial experimentation, causing great reductions in the MS/MS response. Our approach to resolve this suppression involved modifying the chromatographic conditions so that 25(OH) D_2 , 25(OH) D_3 , and Δ^9 -THC- D_3 co-eluted. Indeed, the latter approach compensated for any matrix ion suppression without affecting actual results.

There has been an increased focus on redefining 25(OH)D reference intervals for accurate diagnosis of vitamin D insufficiency and deficiency. Certain populations are at greater risk for hypovitaminosis D, including postmenopausal women, non-Caucasian individuals, people who live in cultures with dress restrictions limiting sun exposure, and breast-fed infants. Epidemiologic studies also have demonstrated strong evidence of an inverse correlation between serum 25(OH)D levels and PTH levels. Secondary hyperparathyroidism and osteoporosis ultimately might develop in patients with persistently deficient levels of 25(OH)D.²²⁻²⁵

Much of the current literature on 25(OH)D levels recommends cutoff values between 20 and 32 ng/mL (50-80 nmol/L) to diagnose vitamin D deficiency.²⁶⁻²⁸ At these higher levels, secondary hyperparathyroidism subsides, bone mineral density increases substantially, optimal intestinal calcium absorption is observed, and nonspecific musculoskeletal chronic pain can subside.^{3,22-25} As awareness of vitamin D deficiency increases, more laboratories are increasing levels used to define desirable and normal levels of total 25(OH)D. Ideally, it is advantageous to have established guidelines for 25(OH)D levels that correspond to disease states owing to the large annual fluctuations in vitamin D levels in many people. Although serum levels of 25(OH)D ideally are suited to best represent a patient's true vitamin D status, measurement of 1,25-dihydroxyvitamin D also is clinically useful to assess alterations in various disorders of calcium metabolism. An LC-MS/MS assay is in development to detect 1,25-dihydroxyvitamin D at picomolar concentrations.

Results from our reference interval study advocate use of 25(OH)D guidelines based on disease states vs reference intervals based on a seemingly healthy population. It is critical to note that these studies were conducted in the middle of winter in Seattle (47° latitude). A minimum energy of 20

kJ/cm² is needed to produce cutaneous vitamin D_3 , and, during the winter months in US states above 40° latitude, that exposure level is not achieved, even during seemingly sunny conditions.^{29,30} Thus, our patient population was theoretically in its lowest annual vitamin D status. Extremely low levels were achieved in the population of healthy volunteers, with the calculated reference interval mean for total 25(OH)D (19.3 ng/mL [48 nmol/L]) only slightly higher than our current desirable level (>15 ng/mL [>37 nmol/L]). However, it would be unwise to decrease our present guidelines owing to the numerous studies documenting impaired endocrine function and increased risk of disease to patients at low circulating 25(OH)D levels. More study is warranted to establish reference intervals that truly represent the healthy population in the Northwest.

We have developed a rapid, sensitive LC-MS/MS method for specifically measuring serum levels of 25(OH) D_2 and 25(OH) D_3 at nanomolar concentrations using a novel internal standard. Owing to lower cost and shorter analysis times, this method is suitable not only for routine clinical measurement of serum 25(OH)D levels but also for multicenter clinical trials.

From the Department of Laboratory Medicine, University of Washington Medical Center and Harborview Medical Center, Seattle.

Address reprint requests to Dr Saenger: Dept of Laboratory Medicine (NW 120; Box 357110), University of Washington Medical Center, 1959 NW Pacific St, Seattle, WA 98195.

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References

1. Vieth R. Vitamin D supplementation, 25-hydroxyvitamin D concentrations, and safety. *Am J Clin Nutr.* 1999;69:842-856.
2. Utiger RD. The need for more vitamin D [editorial]. *N Engl J Med.* 1998;338:828-829.
3. Chapuy MC, Preziosi P, Maamer M, et al. Prevalence of vitamin D insufficiency in an adult normal population. *Osteoporos Int.* 1997;7:439-443.
4. Tangpricha V, Pearce EN, Chen TC, et al. Vitamin D insufficiency among free-living healthy young adults. *Am J Med.* 2002;112:659-662.
5. Hanchette CL, Schwartz GG. Geographic patterns of prostate cancer mortality. *Cancer.* 1992;70:2861-2869.
6. Holick MF. Vitamin D: importance in the prevention of cancers, type 1 diabetes, heart disease, and osteoporosis. *Am J Clin Nutr.* 2004;79:362-371.
7. John EM, Schwartz GG, Dreon DM, et al. Vitamin D and breast cancer risk: the NHANES I epidemiologic follow-up study, 1971-1975 to 1992. *Cancer Epidemiol Biomarkers Prev.* 1999;8:399-406.
8. Krause R, Buhning M, Hopfenmuller W, et al. Ultraviolet B and blood pressure [letter]. *Lancet.* 1998;352:709-710.
9. Al Faraj S, Al Mutairi K. Vitamin D deficiency and chronic low back pain in Saudi Arabia. *Spine.* 2003;28:177-179.

10. Haddad JG Jr, Hahn TJ. Natural and synthetic sources of circulating 25-hydroxyvitamin D in man. *Nature*. 1973;244:515-517.
11. Eisman JA, Shepard RM, DeLuca HF. Determination of 25-hydroxyvitamin D₂ and 25-hydroxyvitamin D₃ in human plasma using high-pressure liquid chromatography. *Anal Biochem*. 1977;90:298-305.
12. Carter GD, Carter R, Jones J, et al. How accurate are assays for 25-hydroxyvitamin D? data from the International Vitamin D External Quality Assessment Scheme. *Clin Chem*. 2004;50:2195-2197.
13. Higashi T, Awada D, Shimada K. Simultaneous determination of 25-hydroxyvitamin D₂ and 25-hydroxyvitamin D₃ in human plasma by liquid chromatography–tandem mass spectrometry employing derivatization with a Cookson-type reagent. *Biol Pharm Bull*. 2001;24:738-743.
14. Tsugawa N, Suhara Y, Kamao M, et al. Determination of 25-hydroxyvitamin D in human plasma using high-performance liquid chromatography–tandem mass spectrometry. *Anal Chem*. 2005;77:3001-3007.
15. Vogeser M, Kyriatsoulis A, Huber E, et al. Candidate reference method for the quantification of circulating 25-hydroxyvitamin D₃ by liquid chromatography–tandem mass spectrometry. *Clin Chem*. 2004;50:1415-1417.
16. Maunsell Z, Wright DJ, Rainbow S. Routine isotope-dilution liquid chromatography–tandem mass spectrometry assay for simultaneous measurement of the 25-hydroxy metabolites of vitamins D₂ and D₃. *Clin Chem*. 2005;51:1683-1690.
17. National Committee for Clinical Laboratory Standards. How to define and determine reference intervals in the clinical laboratory; approved guideline. C28-A2. 2nd ed. Wayne, PA: NCCLS; 2000.
18. Stamp TB, Round JM. Seasonal changes in human plasma levels of 25-hydroxyvitamin D. *Nature*. 1974;247:563-565.
19. Lissner D, Mason RS, Posen S. Stability of vitamin D metabolites in human blood serum and plasma [letter]. *Clin Chem*. 1981;27:773-774.
20. Hollis BW. The determination of circulating 25-hydroxyvitamin D: no easy task [editorial]. *J Clin Endocrinol Metab*. 2004;89:3149-3151.
21. Annesley TM. Ion suppression in mass spectrometry. *Clin Chem*. 2003;49:1041-1044.
22. Bischoff-Ferrari H, Dietrich T, Orav E, et al. Positive association between 25(OH)D levels and bone mineral density: a population-based study of younger and older adults. *Am J Med*. 2004;116:634-639.
23. Heaney R, Dowell M, Hale C, et al. Calcium absorption varies within the reference range for serum 25-hydroxyvitamin D. *J Am Coll Nutr*. 2003;22:142-146.
24. Lips P, Duong T, Oleksik A, et al. A global study of vitamin D status and parathyroid function in postmenopausal women with osteoporosis: baseline data from the multiple outcomes of raloxifene evaluation clinical trial. *J Clin Endocrinol Metab*. 2001;86:1212-1221.
25. Vieth R, Ladak Y, Walfish P. Age-related changes in the 25-hydroxyvitamin D versus parathyroid hormone relationship suggest a different reason why older adults require more vitamin D. *J Clin Endocrinol Metab*. 2003;88:185-191.
26. Hollis BW. Circulating 25-hydroxyvitamin D levels indicative of vitamin D sufficiency: implications for establishing a new effective dietary intake recommendation for vitamin D. *J Nutr*. 2005;135:317-322.
27. Heaney RP. Vitamin D: how much do we need, and how much is too much? *Osteoporos Int*. 2000;11:553-555.
28. Malabanan A, Veronikis IE, Holick MF. Redefining vitamin D insufficiency [letter]. *Lancet*. 1998;351:805-806.
29. Webb AR, Kline L, Holick MF. Influence of season and latitude on the cutaneous synthesis of vitamin D₃ synthesis in human skin. *Arch Dermatol*. 1988;67:373-378.
30. Matsuoka LY, Wortsman J, Haddad JG, et al. In vivo threshold for cutaneous synthesis of vitamin D₃. *J Lab Clin Med*. 1989;114:301-305.