Rapid Analysis of 25-Hydroxyvitamin D<sub>2</sub> and D<sub>3</sub> by Liquid Chromatography–Tandem Mass Spectrometry and Association of Vitamin D and Parathyroid Hormone Concentrations in Healthy Adults

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Key Words: 25-Hydroxyvitamin D; Mass spectrometry; Parathyroid hormone

Abstract

Measurement of 25-hydroxyvitamin D (25OH-vitD) is used to assess vitamin D status. We developed a high-sensitivity measurement method for 25OH-vitD and assessed the relationship between 25OH-vitD and parathyroid hormone (PTH) in healthy adults. Aliquots (100 µL) of serum were spiked with internal standard, proteins were precipitated, and samples were analyzed by liquid chromatography–tandem mass spectrometry using 2-dimensional chromatographic separation. Total imprecision was less than 10%, and the limit of quantitation was 1.0 ng/mL. We determined the distribution of concentrations of 25OH-vitD<sub>2</sub> and 25OH-vitD<sub>3</sub> in healthy adults using samples collected during winter and summer and evaluated the association between 25OH-vitD and PTH. The difference between median concentrations of 25OH-vitD in samples collected during winter and summer was 11 ng/mL (27 nmol/L). Statistically significant differences in concentrations of PTH were observed between groups of samples with 25OH-vitD less than 11 (27 nmol/L) and 11 to 15 ng/mL (27-37 nmol/L) and between groups with 25 to 30 (62-75 nmol/L) and more than 40 ng/mL (100 nmol/L). Among the advantages of this method are its high sensitivity and specificity.

Vitamin D is a fat-soluble vitamin that is involved in calcium and phosphorus homeostasis. Vitamin D deficiency is associated with osteoporosis and osteomalacia in adults and rickets in children. Recent studies of the physiologic effects of vitamin D suggest its role in autoimmune diseases, cancers, hypertension, multiple sclerosis, and cognitive impairment. Vitamin D naturally occurs in 2 forms: D<sub>2</sub> (ergocalciferol), from plant and fungal sources, and D<sub>3</sub> (cholecalciferol), produced in skin with ultraviolet exposure and from animal sources. Both forms of vitamin D are prohormones that undergo hepatic hydroxylation to 25-OH vitamin D<sub>2</sub> (25OH-vitD<sub>2</sub>) and 25-OH vitamin D<sub>3</sub> (25OH-vitD<sub>3</sub>). A renal 1-α-hydroxylase converts 25OH-vitD to 1,25-dihydroxyvitamin D. Among the metabolites of vitamin D, 25OH-vitD<sub>2</sub> and 25OH-vitD<sub>3</sub> have the highest blood concentration and longest half-lives; because of this fact, 25OH-vitD<sub>2</sub> and 25OH-vitD<sub>3</sub> are considered the best markers of vitamin D status.

Measurements of 25OH-vitD are commonly performed in patients with osteoporosis, osteomalacia, and abnormal concentrations of calcium and phosphorus, and elevated concentrations of parathyroid hormone (PTH). Vitamin D is also measured in patients with malabsorption of vitamin D, celiac disease, cystic fibrosis, and Crohn disease. Current methods for measurement of 25OH-vitD include radioimmunoassay, automated immunochemiluminometric assay (ICMA), high-performance liquid chromatography (HPLC), and liquid chromatography–tandem mass spectrometry (LC-MS/MS). Lack of standard reference materials and variation in assay specificity has led to large interlaboratory differences. LC-MS/MS methods provide better specificity and allow separate quantitation of 25OH-vitD<sub>2</sub> and 25OH-vitD<sub>3</sub> and potentially fewer interlaboratory differences.
The objectives of this study were to develop a highly sensitive and specific method for 25OH-vitD₂ and 25OH-vitD₃, to determine concentrations of 25OH-vitD in the serum of healthy adults in samples collected during winter and summer and to assess the relationship between the concentrations of 25OH-vitD and PTH in healthy subjects.

Materials and Methods

Standards and Reagents

Standard 25OH-vitD₂ was purchased from IsoSciences, King of Prussia, PA, and 25OH-vitD₃ was purchased from USP, Rockville, MD. Deuterated analogs, d₆-25OH-vitD₂ and d₆-25OH-vitD₃, were purchased from Medical Isotopes, Pelham, NH. Stock standards were prepared at a concentration of 1 µg/mL; a working calibration standard was prepared at 100 ng/mL; and internal standards were prepared at concentrations of 60 and 40 ng/mL for d₆-25OH-vitD₂ and d₆-25OH-vitD₃, respectively. All standards were prepared in methanol. Calibration standards were prepared in 1% bovine serum albumin (BSA) at concentrations of 5, 10, 25, 50, and 100 ng/mL. HPLC-grade methanol and acetonitrile were obtained from VWR, West Chester, PA. All other reagents were purchased from Sigma, St Louis, MO, and were of the highest purity commercially available. Certified reference materials were purchased from the National Institute for Standards and Technology (NIST; SRM 972, Gaithersburg, MD) and Polygen (ClinCheck, Gliwice, Poland).

Sample Preparation

Aliquots of 100 µL of standards, controls, or patient samples were transferred into Sirocco 96-well protein precipitation plates (Waters, Milford, MA) containing 300 µL of acetonitrile. To each well were added 50 µL of working internal standard, and the plate was covered. The filtration plate was “vortexed” for 2 minutes on a plate shaker (Barnstead International, Dubuque, IA), held at room temperature for 30 minutes, and filtered into a collection plate by centrifugation (2,000 relative centrifugal force for 3 minutes); the filtrates were partially evaporated (10 minutes at 45°C) using nitrogen, and then 100 µL of water was added to each well. The plate was vortex-mixed, and the samples were analyzed.

Liquid Chromatography–Tandem Mass Spectrometry

The instrument consisted of an API 4000 triple-quadrupole mass spectrometer (Applied Biosystems/MDS Scieix, Concord, Canada). The system included a binary and a quaternary HPLC pump and oven (series 1200, Agilent, Santa Clara, CA) and an HTC PAL autosampler (LEAP Technologies, Carrboro, NC) equipped with a fast-wash station and 6-port switching valve. The first-dimension separation was on a 50 × 2.0-mm chromatographic column, Zorbax Eclipse XDB-CN, with 5-µm particles (Agilent), and the analytical separation was on a 50 × 2.0-mm HPLC column, Gemini C18, with 3-µm particles (Phenomenex, Torrance, CA).

The injection volume was 150 µL, and the oven temperature was 35°C. The mobile phase consisted of 10 mmol/L aqueous formic acid and methanol containing 10 mmol/L formic acid. The mobile phase for the first-dimension separation was delivered at a flow rate of 0.7 mL/min with a gradient: 20% methanol for 0.2 minute, linear gradient between 0.2 and 2.5 minutes to 75% methanol, held for 2.5 minutes, fast gradient to 95%, held for 2 minutes, followed by reequilibration to initial conditions. Mobile phase for the analytic separation was delivered at a flow rate of 0.6 mL/minute with a gradient: 75% methanol for 2.6 minutes, linear gradient between 3.0 and 3.2 minutes to 95% methanol, held for 2.5 minutes, followed by reequilibration to initial conditions between injections. A switching valve was installed between the columns; effluent from the CN column was directed to the analytic column from 2.1 to 2.6 minutes.

The autosampler injection syringe was washed between injections 2 times with a methanol-water mix (ratio, 4:1) containing 20 mmol/L of trifluoroacetic acid and 2 times with 2-propanol. The quadrupoles Q1 and Q3 were tuned to unit resolution, and the mass spectrometer conditions were optimized for maximum signal intensity of 25OH-vitD₂ and 25OH-vitD₃. The instrument was used with an atmospheric pressure chemical ionization ion source operated at 250°C in positive-ion mode; the declustering potential was 50 V, and the entrance potential was 10V; nitrogen was used as the collision gas. Two mass transitions were monitored for each analyte and the internal standard. The primary mass transitions were a mass/charge ratio (m/z) of 413 to 337 (collision energy, 17 V) for 25OH-vitD₂ and an m/z of 401 to 365 (collision energy, 17 V) for 25OH-vitD₃; the secondary mass transitions were the same as the primary mass transitions but acquired at a collision energy of 11 V. Corresponding mass transitions of internal standards were an m/z of 419 to 337 for d₆-25OH-vitD₂ and an m/z of 407 to 371 for d₆-25OH-vitD₃. All data were acquired and processed with Analyst 1.4.2 software (Applied Biosystems/MDS Scieix). Calibration was performed with every batch of samples.

Assay Performance Characteristics

Evaluation of method performance included imprecision, limit of detection (LOD), limit of quantitation (LOQ), upper limit of linearity, method comparison, recovery, carryover, and ion suppression. Imprecision was determined by analyzing 3 replicates per run of human plasma samples containing 25OH-vitD₂ and 25OH-vitD₃ at concentrations ranging between 4 and 185 ng/mL (10-462 nmol/L) in 1 run per day over 6 days and 2 commercial control samples analyzed in
Results

Chromatograms of 2 multiple reaction monitoring transitions of 25OH-vitD₂ and 25OH-vitD₃ are shown Figure 1. The LOD and LOQ for 25OH-vitD₂ and 25OH-vitD₃ were determined to be 0.5 and 1.0 ng/mL, respectively. The method was linear to 1,000 ng/mL (2,496 nmol/L) for 25OH-vitD₂ and 25OH-vitD₃.

The stability of 25OH-vitD₂ and 25OH-vitD₃ was evaluated in methanol (100 ng/mL) and in human plasma under different storage conditions. Aliquots of plasma containing 30 ng/mL (75 nmol/L) of 25OH-vitD₂ and 25OH-vitD₃ were stored at room temperature, in a refrigerator (4°C), and in a freezer (−20°C). The samples were transferred into a −70°C freezer after 1, 3, 7, 14, 21, and 28 days of storage and analyzed in a single batch. Assessment of specimen suitability was performed by analyzing blood from 16 people collected in lithium heparin, sodium heparin, sodium citrate, potassium EDTA, serum, plasma separator, and serum separator tubes.

Samples From Healthy Subjects

We collected serum samples from self-reported healthy volunteers (office workers located at 40° north, 111° west; average elevation, 1,320 m) for assessment of the distribution of concentrations of 25OH-vitD and PTH. A set of 130 samples was collected in winter (February; 72 samples from women and 58 from men), and another set of 130 samples was collected in summer (July-August; 77 samples from women and 53 from men). Serum was separated from RBCs within 1 hour of collection, and the samples were stored at −70°C before the analysis. The median age of men was 31 years (range, 19-59 years), and the median age of women was 30 years (range, 19-65 years). More than 90% of the participants were white, and more than 96% were nonsmokers. Samples were analyzed for PTH, calcium, and creatinine (using Modular Analytics P 800 and E170 module analyzers, Roche, Indianapolis, IN), and the estimated glomerular filtration rate was calculated using the Modification of Diet in Renal Disease equation.28 People with abnormal calcium results or an estimated glomerular filtration rate less than 60 mL/min/1.73 m² were excluded from the study. All studies with samples from human subjects were approved by the University of Utah Institutional Review Board (Salt Lake City); informed consent was obtained from all participants.

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respectively. Within-run, between-run, and total imprecision data are shown in Table 1. The results of method comparison with commercial immunoassays and LC-MS/MS method of a commercial laboratory are shown in Figure 2. Our method agreed well with the other LC-MS/MS method, whereas large scatter was observed in the comparison for the total concentration of 25OH-vitD (25OH-vitD₂ + 25OH-vitD₃) with ICMA. The accuracy of our LC-MS/MS method was evaluated using NIST standards (SRM 972). The measured concentrations of 25OH-vitD₂ and 25OH-vitD₃ in 3 SRM samples were within 2% to 10% of the target values. We did not observe peaks coeluting with 25OH-vitD₂ or 25OH-vitD₃ in patient samples. Assessment of the specificity for 25OH-vitD₂ and 25OH-vitD₃ in patient samples was performed through evaluation of the ratios of concentrations determined from the 2 sets of mass transitions of the analytes and the internal standards.

Evaluation of the stability of 25OH-vitD₂ and 25OH-vitD₃ in plasma samples indicated good stability under the conditions evaluated. There was no evidence of degradation of 25OH-vitD₂ and 25OH-vitD₃ after 3 freeze-thaw cycles. In samples prepared in methanol, we observed partial degradation of 25OH-vitD₂ and 25OH-vitD₃ after storage at room and refrigerated temperatures. Degradation products were partially resolved from the peaks of 25OH-vitD₂ and 25OH-vitD₃, and their intensities increased after extended storage at room and refrigerated temperatures and exposure to light.

To determine the adequacy of vitamin D in a local population and to evaluate the association between concentrations of 25OH-vitD and PTH, we analyzed samples from apparently healthy adult volunteers. The cumulative distribution of concentrations of 25OH-vitD, PTH, and the ratios of 25OH-vitD/PTH are shown in Figure 3. A median concentration of

<table>
<thead>
<tr>
<th>Sample</th>
<th>Mean, ng/mL (nmol/L)</th>
<th>Within-Run CV (%)</th>
<th>Between-Run CV (%)</th>
<th>Total CV (%)</th>
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<tr>
<td>25-Hydroxyvitamin D₂</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Level 1*</td>
<td>10.2 (25)</td>
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<tr>
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<td>7.6</td>
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<tr>
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<td>Level 6*</td>
<td>148.0 (370)</td>
<td>4.5</td>
<td>1.2</td>
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<tr>
<td>Level 7†</td>
<td>186.0 (464)</td>
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<td>7.7</td>
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<tr>
<td>25-Hydroxyvitamin D₃</td>
<td></td>
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</tr>
<tr>
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<td>3.8 (9)</td>
<td>6.0</td>
<td>4.0</td>
<td>7.2</td>
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<tr>
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<td>97.8 (236)</td>
<td>4.4</td>
<td>2.0</td>
<td>4.8</td>
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CV, coefficient of variation.
* Analyzed in triplicate over 6 days.
† Analyzed in duplicate over 20 days.
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25OH-vitD₂ and the central 95% of the distribution were 0.7 ng/mL (2 nmol/L) and less than 1 to 6.3 ng/mL (2-16 nmol/L), respectively. The results were evaluated for seasonal and between-sex differences in the concentrations of 25OH-vitD, PTH, and the ratio 25OH-vitD/PTH (Figure 3). An association between the concentrations of 25OH-vitD and PTH was evaluated for the entire data set and for the median values of the data grouped by the ranges of the 25OH-vitD concentrations (Figure 4). Paired comparisons between the groups were performed with the Wilcoxon nonparametric test (JMP4 software, SAS Institute, Cary, NC). The median concentrations of PTH were statistically significantly different between groups with 25OH-vitD concentrations less than 11 ng/mL (27 nmol/L) and 11 to 15 ng/mL (27-37 nmol/L; \( P = .003 \)). The difference approached statistical significance between the groups with concentrations of 25OH-vitD of 26 to 30 ng/mL (65-75 nmol/L) and 31 to 35 ng/mL (77-87 nmol/L; \( P = .060 \)) and was statistically significant between the groups with concentrations of 25 to 30 (62-75 nmol/L) and more than 40 ng/mL (100 nmol/L; \( P = .003 \)).

Discussion

LC-MS/MS is considered the most accurate technique for analysis of 25OH-vitD. LC-MS/MS methods typically use sample pretreatment to separate the 25OH-vitD from its binding protein followed by simultaneous measurement of 25OH-vitD₂ and 25OH-vitD₃. The majority of published methods for 25OH-vitD use dehydrated parent ions or loss of water as product ions. Even though these methods provide accurate quantitation of 25OH-vitD, the choice of

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**Figure 2** Bland-Altman plots for liquid chromatography–tandem mass spectrometry (LC-MS/MS) method comparison with a commercial laboratory LC-MS/MS method for 25-hydroxyvitamin D₂ (A), 25-hydroxyvitamin D₃ (B), total 25-hydroxyvitamin D (C), and a commercial chemiluminescent method (D). Deming regression equations for the comparison with the LC-MS/MS method were LC-MS/MS\textsubscript{eval} = 1.03 \times \text{LC-MS/MS}_{\text{ref}} - 0.5 (r = 0.991; \text{Sy/x} = 1.66) and LC-MS/MS\textsubscript{eval} = 1.045 \times \text{LC-MS/MS}_{\text{ref}} - 1.1 (n = 91; r = 0.960; \text{Sy/x} = 3.2), for 25-hydroxyvitamin D₂ and 25-hydroxyvitamin D₃, respectively. Deming regression equation for comparison with the immunochemiluminescent assay (ICMA) was ICMA = 1.09 \times \text{LC-MS/MS}_{\text{eval}} - 2.4; n = 371; r = 0.788; \text{Sy/x} = 6.8. Values are given in conventional units; to convert to Système International units (nmol/L), multiply by 2.496. eval, evaluated method; ref, reference method.
Figure 3: Cumulative frequency of the seasonal distribution of concentrations of total 25-hydroxyvitamin D (25OH-vitD; measured by liquid chromatography–tandem mass spectrometry) (A), parathyroid hormone (PTH) (B), 25OH-vitD/PTH ratio (C), and concentrations of 25OH-vitD grouped by sex in samples from healthy adults (D). Values for 25OH-vitD are given in conventional units; to convert to Système International (SI) units (nmol/L), multiply by 2.496.

Figure 4: Association between concentrations of 25-hydroxyvitamin D (25OH-vitD) and parathyroid hormone (PTH) in the serum of healthy adults (A) and median concentrations and central 90th percent of the distribution of PTH concentrations depending on the concentration of 25OH-vitD (B). Values for 25OH-vitD are given in conventional units; to convert to Système International (SI) units (nmol/L), multiply by 2.496.
parent and product ions affects the background noise and, consequently, the sensitivity and specificity of the methods. In the past, methods for measurement of 25OH-vitD were poorly harmonized, and there were no reference materials for 25OH-vitD available. In contrast with earlier published methods, our method is more sensitive and uses protonated molecular ions, product ions other than the loss of a single water molecule as the mass transitions, high efficiency 2-dimensional (2D) chromatographic separation, and assessment of specificity through monitoring of multiple mass transitions.

Ionization of 25OH-vitD using atmospheric pressure chemical ionization was considerably more efficient than electrospray ionization. Protonated molecular ions of 25OH-vitD2 and 25OH-vitD3 were observed at a low ion source temperature; the intensity was substantially reduced as the ion source temperature increased to more than 300°C. The choice of the mass transitions m/z of 413 to 337 (25OH-vitD3) and m/z of 401 to 365 (25OH-vitD2) was based on greater specificity and lower background noise. The commonly used single water loss product ions were not used in the method because they produced high background noise with consequent lower sensitivity.

This method uses a 2D chromatographic separation with a CN column for the first dimension, followed by separation on the reverse phase analytic column. The CN column resolved 25OH-vitD from the majority of the sample constituents, while peaks of 25OH-vitD2 and 25OH-vitD3 simultaneously eluted onto the reverse phase column for separation from hydrophobic compounds that coeluted with 25OH-vitD on the CN column. The ratio of the concentrations of the primary and the secondary mass transitions was used to ensure analytic specificity.

Chemical properties of 25OH-vitD3 and 25OH-vitD2 are very similar; however, we observed differences in the ionization efficiency and chromatographic retention between the compounds. As chromatographic columns aged, an increase in the peak width was observed for 25OH-vitD3 but not for 25OH-vitD2. Considering differences in chromatographic retention and the ionization, use of 2 isotopically labeled internal standards is necessary for reliable quantitation of 25OH-vitD2 and 25OH-vitD3.

Assessment of the methods’ performance in routine use was performed with 2 commercial control materials (ClinChek). This method resolved isobaric peaks from the peaks of 25OH-vitD2 and 25OH-vitD3 in the ClinChek control materials. Peaks at this RT were not present in any of the patient samples but were observed in the standards of 25OH-vitD2 and 25OH-vitD3 prepared in methanol after storage at ambient and refrigerated conditions. This finding suggests an exogenous origin of these compounds. Resolving the degradation products from 25OH-vitD2 and 25OH-vitD3 in ClinChek control materials resulted in lower than expected concentrations in these controls.

The chromatographic conditions used in this method do not resolve C1-epi-25OH isomers of vitamin D3 and vitamin D2 from 25OH-vitD2 and 25OH-vitD3; therefore, this method is not suitable for testing 25OH-vitD in samples from infants. Comparison of this method with a commercial LC-MS/MS method for 25OH-vitD2 and total 25OH-vitD showed good agreement (Figure 2), whereas discordance was observed for 25OH-vitD2 at concentrations less than 5 ng/mL (12 nmol/L). This discordance was likely related to lower sensitivity of the comparison method: the LOQ of this method is 1 ng/mL, whereas the LOQ of the comparison method is 4 ng/mL. Use of the LOQ of 4 ng/mL would result in underestimation of the concentration of 25OH-vitD in the samples in which 25OH-vitD2 is present at concentrations between 1 and 4 ng/mL (2-10 nmol/L). This may be an explanation of better agreement among the methods for 25OH-vitD2 than for 25OH-vitD (Figure 2).

Comparison with a commercial ICMA showed substantial discrepancy between the methods (Figure 2). These observations were consistent with the results of the comparison between ICMA and LC-MS/MS reported by Roth et al. Possible explanations for the large scatter when compared with ICMA are the cross-reactivity between 25OH-vitD and other hydroxy metabolites of vitamin D and differences between calibration across multiple analyzers (ICMA measurements were performed on multiple instruments). Interindividual differences in vitamin D binding protein concentrations and binding affinities could also contribute to matrix effects with the ICMA measurements.

For LC-MS methods, it is important to determine whether recovery is related to the sample preparation or to matrix effects because both can lead to inaccurate measurements. Lower absolute recovery in samples spiked before filtration suggests adsorption of the 25OH-vitD2 and 25OH-vitD3 by the filtration plate; higher concentrations in the samples spiked after filtration suggest loss of the deuterium-labeled internal standards while samples were in the filtration plate. Relative recovery of the analytes was not affected by adsorption because of the use of stable isotope–labeled internal standards added at beginning of the sample preparation. The observed difference in the recoveries between 25OH-vitD2 and 25OH-vitD3 supports the use of individual internal standards for 25OH-vitD2 and 25OH-vitD3. Evaluation of the matrix effects showed no ion suppression at the RT of the 25OH-vitD2 and 25OH-vitD3. Negative deflection in the baseline was observed after 25OH-vitD2 and 25OH-vitD3 eluted from the chromatographic column.

Stability of 25OH-vitD2 and 25OH-vitD3 stored in methanol and in plasma samples was evaluated. Results of the evaluation indicated partial degradation of the standards.
prepared in methanol occurring on storage at room temperature at a rate of approximately 2% per week. No reduction in the concentrations of 25OH-vitD$_2$ and 25OH-vitD$_3$ (nor presence of the degradation product) was observed in plasma samples stored at all conditions we evaluated; one explanation of better stability of 25OH-vitD in plasma samples is the presence of vitamin D binding proteins.

No statistically significant differences were observed in concentrations of 25OH-vitD$_2$ and 25OH-vitD$_3$ between plasma and serum samples obtained from 16 people in a variety of collection tubes, with the exception of sodium citrate tubes. Concentrations of 25OH-vitD$_2$ and 25OH-vitD$_3$ in sodium citrate samples were approximately 15% lower compared with the other 6 types of collection tubes.

Because concentrations of 25OH-vitD in blood are not only associated with the status of health, but also depend on the geographic location, lifestyle, and diet, a reference interval for 25OH-vitD cannot be established by using approaches commonly used for other endogenous biomarkers. An indirect way to assess the physiologic sufficiency of vitamin D is through the evaluation of the association between concentrations of PTH and 25OH-vitD. PTH is known to be elevated in people with vitamin D deficiency and shows a tendency to decrease as concentrations of 25OH-vitD increase.$^{31-34}$ This well-known inverse relationship between PTH and 25OH-vitD was confirmed in this study by using serum samples from healthy adults (Figure 4). Evaluation of the median concentrations of PTH in the samples grouped by ranges of 25OH-vitD demonstrated significant differences in the concentrations of PTH between samples with 25OH-vitD concentrations 11 ng/mL (27 nmol/L) or less and 11 to 15 ng/mL (27-37 nmol/L). The difference approached statistical significance between the groups with concentrations of 25OH-vitD of 26 to 30 ng/mL (65-75 nmol/L) and 31 to 35 ng/mL (77-87 nmol/L) and was also statistically significant between the groups with concentrations of 25 to 30 (62-75 nmol/L) and greater than 40 ng/mL (100 nmol/L). The regression line of the dependence between concentrations of PTH and 25OH-vitD flattened at concentrations of greater than 30 ng/mL (75 nmol/L), although a trend of continuing decline in values of PTH was observed in groups with higher concentrations of 25OH-vitD. These data support a cutoff of 30 ng/mL (75 nmol/L) of 25OH-vitD as the lower boundary of the reference interval, while the data also demonstrate a trend for a continuing reduction of PTH in samples with concentrations of 25OH-vitD of greater than 30 ng/mL (75 nmol/L).

Substantial differences in 25OH-vitD concentrations in samples collected from healthy volunteers in summer and winter were observed (Figure 3); the mean value of the seasonal difference was 11 ng/mL (27 nmol/L). In samples collected in summer and in winter, concentrations of 25OH-vitD were less than 30 ng/mL (75 nmol/L) in 39% and 78% of samples, respectively. A less distinct effect of the seasonal variation was observed in the concentrations of PTH, whereas the ratios 25OH-vitD/PTH showed substantial seasonal differences. Because of the inverse relationship between concentrations of PTH and 25OH-vitD, the ratio 25OH-vitD/PTH may serve as a better indicator of the status of vitamin D than PTH or 25OH-vitD alone (Figure 3). Cumulative distribution of the concentrations of 25OH-vitD showed substantial differences in the concentrations between genders (Figure 3). The difference may be related to lifestyle or possible gender-specific physiology. Unfortunately, we do not have information on vitamin D supplement intake by our subjects and presume that they are representative of the US population at large.

We have developed a sensitive and specific method for quantitation of 25OH-vitD$_2$ and 25OH-vitD$_3$ in human serum that uses 2D chromatographic separation and MS/MS detection. By avoiding the use of the dehydrated ions of 25OH-vitD$_2$ and 25OH-vitD$_3$ as the molecular and product ions, this method uses more specific multiple reaction monitoring transitions that provide greater sensitivity and specificity. Performance was assessed by comparison with a commercial laboratory LC-MS/MS method, ICMA, and commercial controls from NIST and Polygen. Results of the comparison with another LC-MS/MS method suggest the need to have an LOQ for 25OH-vitD$_2$ and 25OH-vitD$_3$ of 1 ng/mL. Our data indicate that measurements of 25OH-vitD with LC-MS/MS are more specific than with ICMA. Substantial differences in concentrations of 25OH-vitD in samples collected from healthy adults were observed between summer and winter, whereas less seasonal variation was observed for PTH. Because of the inverse relationship between concentrations of PTH and 25OH-vitD, the ratio of 25OH-vitD/PTH may serve as a better indicator of the status of vitamin D than 25OH-vitD measurement alone.

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