Rapid Analysis of 25-Hydroxyvitamin D$_2$ and D$_3$ by Liquid Chromatography–Tandem Mass Spectrometry and Association of Vitamin D and Parathyroid Hormone Concentrations in Healthy Adults

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A b s t r a c t

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$_2$ and 25OH-vitD$_3$ 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.$^{1-3}$ Recent studies of the physiologic effects of vitamin D suggest its role in autoimmune diseases,$^{4-6}$ cancers,$^{7-10}$ hypertension,$^{11,12}$ multiple sclerosis,$^{13,14}$ and cognitive impairment.$^{15,16}$ Vitamin D naturally occurs in 2 forms: D$_2$ (ergocalciferol), from plant and fungal sources, and D$_3$ (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$_2$ (25OH-vitD$_2$) and 25-OH vitamin D$_3$ (25OH-vitD$_3$). A renal 1-α-hydroxylase converts 25OH-vitD to 1,25-dihydroxyvitamin D. Among the metabolites of vitamin D, 25OH-vitD$_2$ and 25OH-vitD$_3$ have the highest blood concentration and longest half-lives; because of this fact, 25OH-vitD$_2$ and 25OH-vitD$_3$ 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.$^{17,18}$ Current methods for measurement of 25OH-vitD include radioimmunooassay, automated immunochemiluminometric assay (ICMA), high-performance liquid chromatography (HPLC), and liquid chromatography–tandem mass spectrometry (LC-MS/MS).$^{19,20}$ Lack of standard reference materials and variation in assay specificity has led to large interlaboratory differences.$^{21-24}$ LC-MS/MS methods provide better specificity and allow separate quantitation of 25OH-vitD$_2$ and 25OH-vitD$_3$ 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 Siroc 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 Sciex, 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.25 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 Sciex). 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
duplicate over 20 days. Linearity was evaluated by analyzing 7 samples with 25OH-vitD2 and 25OH-vitD3 concentrations between 20 and 1,000 ng/mL (50-2,496 nmol/L). Samples for evaluation of linearity were prepared in 0.1% BSA dissolved in phosphate-buffered saline. Two standards containing 25OH-vitD2 and 25OH-vitD3 at concentrations of 20 ng/mL (50 nmol/L) and 1,000 ng/mL (2,496 nmol/L) were prepared and mixed in different proportions. Method LOD and LOQ were determined by analyzing 6 samples containing progressively lower concentrations of 25OH-vitD2 and 25OH-vitD3 down to 0.2 ng/mL (0.5 nmol/L). A sample containing 25OH-vitD2 and 25OH-vitD3 at concentrations higher than the lowest calibrator was analyzed, and then additional samples were prepared by serial dilution (×2) of the aforementioned sample using 0.1% BSA in phosphate-buffered saline. Samples for evaluation of the linearity and sensitivity were analyzed in duplicate over 3 days.

Criteria of maintaining accuracy within ±15%, imprecision (coefficient of variation) less than 20%, and a branching ratio of the mass transitions within ±20% were used to determine the upper limit of linearity and LOQ for the assay. Assessment of the specificity in unknown samples was performed by evaluating the branching ratio of the mass transitions; values outside of the ±20% range were considered to indicate interference. The LOD was determined as the lowest concentration at which chromatographic peaks of the 25OH-vitD2 and 25OH-vitD3 were present in both transitions at expected retention times (RT) with a signal/noise ratio of more than 10. Recovery of the method was evaluated by comparing concentrations of 25OH-vitD2 and 25OH-vitD3 in 5 patient samples analyzed as is and after addition of 25 ng/mL of 25OH-vitD2 and 25OH-vitD3 before and after filtration; the experiment was performed twice on separate days.

To evaluate the robustness of the method, we analyzed more than 3,000 patient samples. A ratio of concentrations determined from different mass transitions greater than ±20%, broadening of chromatographic peaks, split peaks, or an increase in the background were interpreted as potential interference. Ion suppression was evaluated by analyzing patient samples with concentrations of 25OH-vitD2 and 25OH-vitD3 less than 20 ng/mL (50 nmol/L) injected in flow of standards of 25OH-vitD2 and 25OH-vitD3 (each at 1 μg/mL) and infused into effluent of the chromatographic column at a flow rate of 0.017 μL/min. A decrease in the intensity of the baseline in the mass transitions of 25OH-vitD indicated ion suppression.

Methods Comparison and Specimen-Type Suitability and Stability

Our LC-MS/MS method was compared with one from a commercial clinical laboratory (n = 61) and an ICMA for 25OH-vitD (n = 371; Liaison analyzer, Diasorin, Stillwater, MN). Samples used for the method comparison were residual serum and plasma samples submitted for routine testing of 25OH-vitD. The results were evaluated by Deming regression. 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. 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.

Results

Chromatograms of 2 multiple reaction monitoring transitions of 25OH-vitD2 and 25OH-vitD3 are shown in Figure 11. 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 (r = 0.998) and 25OH-vitD1 (r = 0.997). The mean recoveries before filtration were 91% and 98% and after filtration were 106% and 109% for 25OH-vitD2 and 25OH-vitD3.
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.

### Table 1

<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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<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>25-Hydroxyvitamin D₂</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Level 1*</td>
<td>10.2 (25)</td>
<td>4.9</td>
<td>3.6</td>
<td>6.1</td>
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<tr>
<td>Level 2*</td>
<td>21.6 (54)</td>
<td>8.7</td>
<td>4.4</td>
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<tr>
<td>Level 3*</td>
<td>25.3 (63)</td>
<td>8.8</td>
<td>3.5</td>
<td>9.5</td>
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<tr>
<td>Level 4*</td>
<td>57.3 (143)</td>
<td>7.7</td>
<td>2.4</td>
<td>7.8</td>
</tr>
<tr>
<td>Level 5*</td>
<td>102.7 (256)</td>
<td>9.1</td>
<td>4.0</td>
<td>10.0</td>
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<tr>
<td>Level 6*</td>
<td>148.4 (370)</td>
<td>4.5</td>
<td>1.2</td>
<td>4.6</td>
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<tr>
<td>Level 7*</td>
<td>186.0 (464)</td>
<td>4.9</td>
<td>5.9</td>
<td>7.7</td>
</tr>
<tr>
<td><strong>25-Hydroxyvitamin D₃</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Level 1*</td>
<td>3.8 (9)</td>
<td>6.0</td>
<td>4.0</td>
<td>7.2</td>
</tr>
<tr>
<td>Level 2*</td>
<td>8.2 (20)</td>
<td>7.0</td>
<td>1.8</td>
<td>7.2</td>
</tr>
<tr>
<td>Level 3*</td>
<td>28.0 (70)</td>
<td>5.4</td>
<td>2.4</td>
<td>5.9</td>
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<tr>
<td>Level 4*</td>
<td>31.2 (78)</td>
<td>3.0</td>
<td>6.2</td>
<td>6.9</td>
</tr>
<tr>
<td>Level 5*</td>
<td>70.3 (175)</td>
<td>3.7</td>
<td>6.4</td>
<td>7.4</td>
</tr>
<tr>
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<td>5.8</td>
<td>5.1</td>
<td>7.7</td>
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<tr>
<td>Level 7*</td>
<td>97.8 (236)</td>
<td>4.4</td>
<td>2.0</td>
<td>4.8</td>
</tr>
</tbody>
</table>

CV, coefficient of variation. * Analyzed in triplicate over 6 days. † Analyzed in duplicate over 20 days.
Kushnir et al / Rapid Analysis of 25-OH Vitamin D

25OH-vitD 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...
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.
Kushnir et al / Rapid Analysis of 25-OH Vitamin D

Published methods,19,29,30 our method is more sensitive and
Kushnir et al / Rapid Analysis of 25-OH Vitamin D

in ClinChek control materials resulted in lower than expected concentrations in these controls.

The chromatographic conditions used in this method do not resolve C3-epi-25OH isomers of vitamin D3 and vitamin D2 from 25OH-vitD3 and 25OH-vitD2; therefore, this method is not suitable for testing 25OH-vitD in samples from infants.29 Comparison of this method with a commercial LC-MS/MS method for 25OH-vitD3 and total 25OH-vitD showed good agreement (Figure 2), whereas discordance was observed for 25OH-vitD3 at concentrations less than 5 ng/mL (12 mmol/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 underes-
timation of the concentration of 25OH-vitD in the samples in which 25OH-vitD3 is present at concentrations between 1 and 4 ng/mL (2-10 mmol/L). This may be an explanation of better agreement among the methods for 25OH-vitD3, than for 25OH-vitD (Figure 2).

Comparison with a commercial ICMA showed sub-
stantial discrepancy between the methods (Figure 2). These observations were consistent with the results of the compari-
son between ICMA and LC-MS/MS reported by Roth et al.22 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). Intermidividual 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 wheth-
er 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 adsorp-
tion 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-vitD3 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<sub>2</sub> and 25OH-vitD<sub>3</sub> (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<sub>2</sub> and 25OH-vitD<sub>3</sub> 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<sub>2</sub> and 25OH-vitD<sub>3</sub> 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. 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 concentrations 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 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 quantification of 25OH-vitD<sub>2</sub> and 25OH-vitD<sub>3</sub> in human serum that uses 2D chromatographic separation and MS/MS detection. By avoiding the use of the dehydrated ions of 25OH-vitD<sub>2</sub> and 25OH-vitD<sub>3</sub> 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<sub>2</sub> and 25OH-vitD<sub>3</sub> 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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