**Background:** The concentration of 25-hydroxyvitamin D (25(OH)D) in serum has been designated the functional indicator of vitamin D (VitD) nutritional status. Unfortunately, variability among 25(OH)D assays limits clinician ability to monitor VitD status, supplementation, and toxicity.

**Methods:** We developed an HPLC method that selectively measures 25-hydroxyvitamin D$_2$ [25(OH)D$_2$] and D$_3$ [25(OH)D$_3$] and compared this assay with a liquid chromatography–tandem mass spectrometry (LC-MS/MS) method, a competitive protein-binding assay (CPBA) on the Nichols Advantage™ platform, and an RIA from Diasorin.

**Results:** For the new HPLC assay, between-run CVs were 2.6%–4.9% for 25(OH)D$_3$ and 3.2%–13% for 25(OH)D$_2$; recoveries were 95%–102%; and the assay was linear from 0.566 g/L to at least 200 g/L. Comparison data were as follows: for HPLC vs LC-MS/MS, $y = 1.01x - 4.82 \mu g/L$ ($S_{y|x} = 4.93 \mu g/L; r = 0.996$) for 25(OH)D$_3$ and $y = 0.902x - 0.566 \mu g/L$ ($S_{y|x} = 2.56 \mu g/L; r = 0.9965$) for 25(OH)D$_2$ for HPLC vs Diasorin RIA, $y = 0.709x - 5.86 \mu g/L$ ($S_{y|x} = 7.35 \mu g/L; r = 0.7509$); and for HPLC vs Nichols Advantage CPBA, $y = 1.00x - 3.60 \mu g/L$ ($S_{y|x} = 32.7 \mu g/L; r = 0.6823$).

**Conclusions:** The new HPLC method is reliable, robust, and has advantages compared with the Nichols Advantage CPBA and the Diasorin RIA. The Nichols Advantage CPBA overestimated or underestimated 25(OH)D concentrations predicated on the prevailing metabolite present in patients’ sera.

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**HPLC Method for 25-Hydroxyvitamin D Measurement: Comparison with Contemporary Assays**

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Vitamin D (VitD) insufficiency/deficiency exists in epidemic proportions in the general population and is manifested by calcium malabsorption, secondary hyperparathyroidism, muscle weakness, and/or osteoporosis or osteomalacia. Low dietary intake of VitD, lack of exposure to sunlight, and other variables are contributing factors. Described as a “pseudo” vitamin, VitD exists in 2 forms: cholecalciferol (vitamin D$_3$) produced in skin via a photochemical reaction with 7-dehydrocholesterol (1); and ergocalciferol (vitamin D$_2$) derived from plants and used as a supplement. The clinical significance of the chemical differences between the 2 forms and their respective metabolites remains unresolved, although one study reported that ergocalciferol is less effective than cholecalciferol in humans (2).

Clinical laboratory scientists have a diverse array of VitD testing methods from which to choose. Many of these assays have been reported in review articles (3–5), the most recent in 2004; however, additional assays have been introduced since that time. Briefly, current competitive protein-binding assays (CPBAs) for 25-hydroxyvitamin D [25(OH)D] are available from Nichols Institute Diagnostics (Nichols Advantage™ platform), from Immunodiagnostik AG as an enzyme immunoassay, and from Biomedica Gruppe on an ELISA platform. Selective antibodies are incorporated in the Diasorin and IDS Ltd. 125I-based RIAs. Nonradioactive detection is also available: IDS uses ELISA; and Diasorin incorporates chemiluminescence on the Liaison™ platform.

Chromatographic methods can effectively separate 25-hydroxyvitamin D$_3$ [25(OH)D$_3$], D$_2$ [25(OH)D$_2$], and other VitD metabolites. Early methods used silica columns for separations with detection by ultraviolet spectrophotom-
etry but later achieved improved stability with reversed-phase HPLC columns, primarily C_{18} columns (6–8). Moreover, HPLC methods often lacked an internal standard (9), measured 25(OH)D_{3} but not 25(OH)D_{2} (10, 11), required gradient elution (12), or analyzed crude serum extracts that, when injected on an HPLC column, compromised resolution and column life (9). Recently, commercial reagent sets that measure 25(OH)D_{3} but not 25(OH)D_{2} have been marketed by Immundiagnostik AG (normal-phase column) and Chromsystems (reversed-phase column).

Highly selective liquid chromatography–tandem mass spectrometry (LC-MS/MS) methods described in the literature include fast atom bombardment LC-MS/MS with Cookson-type reagents (13) and isotope-dilution electrospray LC-MS/MS (14–16), which is an attractive technique because it enables analysis of crude serum extracts within short run times to gain potentially high throughput. To achieve speed, methods may force elution of metabolites and internal standard under essentially one peak. Under these circumstances, ion suppression can be a major problem (17) but can be minimized through the use of deuterated 25(OH)D_{2} and 25(OH)D_{3} as internal standards. Of equal concern is the potential coelution of 25(OH)-epi-D_{3} with 25(OH)D_{3}. Both compounds have the same molecular mass and chemical composition, but only 25(OH)D_{3}, through its 1,25-dihydroxy metabolite, is active in calcium metabolism (18). Without chromatographic resolution of the 2 compounds, the epimer could mistakenly be reported as 25(OH)D_{3}. Although LC-MS/MS can be the superior method, most clinical laboratories hesitate to embrace the technique because of the substantial cost and need for highly trained operators.

Here we describe a selective validated HPLC method for measurement of 25(OH)D_{3} and 25(OH)D_{2}. The method was developed in response to the inconsistency or restrictions of commercial assays (3) and the clinical concern that an individual may have received a diagnosis of low or normal VitD status depending on which analytical method and laboratory are used (19). Additionally, we compare results for patient samples tested by 4 current methods. Physicians, clinical laboratory scientists, and manufacturers of VitD reagents may find this information useful.

**Materials and Methods**

The VitD metabolites 25(OH)D_{3} and 25(OH)D_{2} were obtained from Fluka Chemicals. 25(OH)-epi-D_{3} was kindly supplied by Dr. G. Satyanarayann Reddy (Providence, RI). Laurophenone (99%), ACS reagent-grade acetonitrile (CH_{3}CN), and ethyl acetate were obtained from Fisher. Methanol (HPLC grade) was obtained from Mallinckrodt Chemicals. Ultrapure water (18.2 MΩ·cm) was obtained from a MilliQ water purification system (Millipore). The precipitation reagent contained the internal standard laurophenone (400 μg/L) in CH_{3}CN and was stored in an amber bottle. Strata-X (surface-modified styrene-divinylbenzene resin) 60-mg (1 mL) extraction cartridges were from Phenomenex. An automated extraction instrument, the Gilson ASPEC XL4 (Gilson Instruments), consisted of a 4-syringe pump module and a 4-needle sampler module with four 2-way solvent ports. Areas in the sampler racks were defined as the sample zone, reagent zone, result zone, and a disposable extraction column (DEC) zone. Acetonitrile–water (35:65 by volume) was stored and delivered from tubes within the reagent zone. The main reservoir contained water. The solvent evaporator was a Turbo Vap™ LV (Caliper Life Sciences). Temperature was set at 35 °C, nitrogen flow was adjusted to 10 psi on the instrument gauge, and the typical drying time setting was 25 min. The HPLC unit was an integrated system with a UV3000 detector set at 275 nm, a P4000 pump set at 1.2 mL/min, an AS2000 autosampler, and a SCM1000 solvent system, all from Thermo Separation Products. A silica-saturator column [250 × 4.6 mm (i.d.) stainless steel column; Alltech] packed with ICN silica gel (particle size, 63–100 μm; MP Biochemicals) was installed in the oven between the pump and injector and is necessary here to prevent deterioration of the analytical column (20). The guard column [(12.5 × 4.6 mm (i.d.))] and analytical column [250 × 4.6 mm (i.d.)], both containing 5-μm Stable Bond™ Cyanopropyl (SB-CN), were from Agilent Technologies. All columns were operated at 50 °C. The methanol–water (67:33 by volume) used as mobile phase was filtered and degassed. Three commercial testing methods were part of our comparison studies and included an LC-MS/MS method from Mayo Laboratories (Rochester, MN) (21); a CPBA on the Nichols Advantage platform with chemiluminescence detection, performed in our laboratory in accordance with manufacturer’s instructions; and an RIA from Diasorin with samples tested by Diasorin.

Individual calibrator stock solutions (40 mg/L) of each metabolite were prepared in ethanol, and the concentration was verified on a Beckman DU 7500 spectrophotometer, using molar absorbivities at 265 nm (1-cm path-length) of 19 400 and 18 300 for 25(OH)D_{2} and 25(OH)D_{3}, respectively (5). From these primary stocks, we prepared a dilute combined stock solution of the compounds at 10 000 μg/L each in ethanol, which was stable for at least 1 year at −20 °C. We then prepared multiple working calibrators in the range of 5–200 μg/L for each of the 2 VitD metabolites combined in a drug-free serum pool. The concentrations of endogenous 25(OH)D_{3} and 25(OH)D_{2} present in the pool were taken into account when assigning the final concentration to the calibrator. Serum calibrators were stored frozen at −20 °C in 10-mL glass vials sealed with Teflon-lined caps (Quorpak™; Fisher Scientific) and were stable for at least 6 months.

Controls were prepared and used in the same manner. Commercial lyophilized serum controls were custom-prepared for us by Utak Laboratories, Inc. Reconstituted Utak controls and thawed calibrators/controls were stable for at least 1 month stored at 4 °C.
**Procedure**

To prepare samples, we dispensed 2 mL of precipitation reagent with internal standard into a 13 × 100 mm disposable glass test tube; we then added 1.0 mL of serum (calibrator, control, or patient sample) to the tube without mixing of contents to avoid “balling” of the protein. The tube was allowed to sit for 5 min at room temperature, after which it was vortex-mixed for 10 s to obtain a flocculent precipitate. After another 5-min wait, the tube was vortex-mixed and centrifuged at 2000g for 10 min. The clear supernatant was decanted into a 10 × 75 mm disposable glass test tube, which was then transferred to the sample zone of the ASPEC XL4 and protected from exposure to natural sunlight to prevent degradation of analytes. The extraction conditions are defined in Table 1.

The XL4 processed 4 samples simultaneously and unattended in ~15 min. The unit sequentially conditioned the Strata-X cartridge in the DEC zone with 2.0 mL of CH3CN followed by 2.0 mL of 35:65 CH3CN–water; added 1.0 mL of water to each extract; transferred 3.5 mL of extract mixture to the DEC; rinsed the DEC with 2.0 mL of 35:65 CH3CN–water; and eluted the Strata-X cartridge in the DEC zone with 2.0 mL of CH3CN. The eluate was dried at 35 °C under a stream of nitrogen; the dry extract was then reconstituted with 150 μL of ethyl acetate–CH3CN (5:95 by volume) and vortex-mixed for 5 s. Water (110 μL) was then added to the tube, and the contents were vortex-mixed for 5 s. The sample was centrifuged at 2000g for 10 min to settle the precipitate. The clear liquid was transferred to a glass microvial insert positioned in an amber-colored vial. The sample was capped and placed in the autosampler unit of the HPLC. The extract was stable for at least 3 days at room temperature. The processor software calculated relative retention time for peak identification and peak-height ratio for quantification.

**Results**

Typical HPLC chromatograms of calibrator and patient sample extracts are illustrated in Fig. 1. Late-eluting peaks were observed at 29 and 37 min; however, chromatographic runs can be shortened by overlapping (injecting) samples at intervals, on average, of every 16–17 min. In these conditions, these late-eluting peaks are “placed” in an area in which they do not obstruct important analyte peaks in succeeding chromatograms.

Between-run precision data were calculated from 5 control sera (n = 20 for each). For 25(OH)D3, the mean (SD) and CV were 4.38 (0.55) μg/L (13%), 11.96 (0.53) μg/L (4.5%), 22.0 (1.09) μg/L (4.9%), 59.8 (1.90) μg/L (3.2%), and 88.7 (3.01) μg/L (3.4%). The between-run precision data for 25(OH)D3 were 11.54 (0.98) μg/L (8.5%), 14.26 (0.56) μg/L (3.9%), 24.56 (1.23) μg/L (5.0%), 45.03 (1.16) μg/L (2.6%), and 82.22 (2.72) μg/L (3.3%). Mean (SD) analytical recoveries based on standard addition to patient samples were as follows: for 25(OH)D3, 95.1 (7.6)% over a concentration range of 4.4–102 μg/L; for 25(OH)D2, 101.2 (9.4)% over a concentration range of 3.2–105 μg/L. The method was linear from the lower limit of quantification (LLQ) of 5 μg/L up to at least 200 μg/L for 25(OH)D3, with the following equation for the regression line: $y = 1.00x + 0.087μg/L$ ($r = 0.9972$; n = 29). The assay was linear for 25(OH)D3 from the LLQ (5 μg/L) up to at least 200 μg/L, with the following equation for the regression line: $y = 1.00x - 0.034μg/L$ ($r = 0.9985$; n = 29). In each case, $x$ represents the concentration added to a serum sample and $y$ the analytical result. The lower limit of detection was the lowest concentration that gave a signal-to-noise ratio of 3 or greater, and the LLQ was the lowest concentration that gave a signal-to-noise ratio of 10 or greater. In a precision study (n = 10) using calibrators at concentrations of 4 and 6 μg/L for both 25(OH)D2 and 25(OH)D3 the signal-to-noise ratios were >14 and the CVs were 7.1%–15%. We therefore selected 5 μg/L as the LLQ.

**Interferences**

When we used Vacutainer™ SST tubes (Becton Dickinson) or Vacuette™ tubes (Greiner), the resulting sera contained substances that appeared as chromatographic runs can be shortened by overlapping (injecting) samples at intervals, on average, of every 16–17 min. In these conditions, these late-eluting peaks are “placed” in an area in which they do not obstruct important analyte peaks in succeeding chromatograms.

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**Table 1. Gilson ASPEC XL4 extraction program.**

<table>
<thead>
<tr>
<th>Program command</th>
<th>Solvent</th>
<th>Volume, mL</th>
<th>Aspirate, mL/min</th>
<th>Dispense, mL/min</th>
<th>Equilibrate, min</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Begin loop</td>
<td>NA*</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>2. Rinse needle (inside/outside)</td>
<td>CH3CN</td>
<td>3.0</td>
<td>20</td>
<td>120</td>
<td>0</td>
</tr>
<tr>
<td>3. Condition DEC</td>
<td>CH3CN</td>
<td>2.0</td>
<td>30</td>
<td>5</td>
<td>0.05</td>
</tr>
<tr>
<td>4. Add to DEC</td>
<td>CH3CN–H2O (35:65 by volume)</td>
<td>2.0</td>
<td>40</td>
<td>5</td>
<td>0.1</td>
</tr>
<tr>
<td>5. Dispense into sample</td>
<td>H2O</td>
<td>1.0</td>
<td>NA</td>
<td>60</td>
<td>0</td>
</tr>
<tr>
<td>6. Load DEC</td>
<td>Diluted serum supernatant</td>
<td>3.5</td>
<td>40</td>
<td>2.5</td>
<td>0.4</td>
</tr>
<tr>
<td>7. Rinse needle (inside/outside)</td>
<td>H2O</td>
<td>4.0</td>
<td>NA</td>
<td>120</td>
<td>0</td>
</tr>
<tr>
<td>8. Add to DEC</td>
<td>CH3CN–H2O (35:65 by volume)</td>
<td>2.0</td>
<td>40</td>
<td>10</td>
<td>0.1</td>
</tr>
<tr>
<td>9. Elute/Collect</td>
<td>CH3CN</td>
<td>2.0</td>
<td>6</td>
<td>3</td>
<td>0.1</td>
</tr>
<tr>
<td>10. Rinse needle</td>
<td>Water</td>
<td>4.0</td>
<td>NA</td>
<td>120</td>
<td>0</td>
</tr>
<tr>
<td>11. End loop</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
</tbody>
</table>

* NA, not applicable.
peaks that coeluted and interfered with measurement of the VitD metabolites. Sera from blood collected in red-top tubes with no additives (available from both companies) did not contain these coeluting peaks. The VitD metabolites 1,25-dihydroxyvitamin D3, 24,25-dihydroxyvitamin D3, and 24,26-dihydroxyvitamin D3 eluted before the internal standard and did not interfere (data not shown). The epimer form, 25(OH)-epi-D3, eluted as a discernible shoulder on the front side of the 25(OH)D3 peak on the SB-CN HPLC column. The epimer is present in low amounts, reportedly <10% of the 25(OH)D3 concentration and less than the method LLQ. The epimer is considered to be inactive in calcium metabolism; however, it can influence parathyroid function (18).

Method–comparison studies demonstrated acceptable agreement between patient results from the HPLC and

---

**Fig. 1. Representative HPLC chromatograms.**

(A), late-eluting peaks; (B), calibrator in extracted serum; (C), sample from patient with low 25(OH)D3 treated with vitamin D2; (D), sample from patient with high concentrations of 25(OH)D3.

Int. Std., internal standard; mAU, milliabsorbance units.

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**Fig. 2. Comparison of the proposed HPLC method with LC/MS/MS.**

**25(OH)D2**
- n = 18
- r = 0.9965
- y = 0.902x - 0.66 μg/L
- $S_{y|x} = 2.56 \, \mu g/L$

**25(OH)D3**
- n = 23
- r = 0.9867
- y = 1.01x - 4.82 μg/L
- $S_{y|x} = 4.93 \, \mu g/L$

---
the LC-MS/MS assays for both 25(OH)D₃ and 25(OH)D₂ (Fig. 2). Conversely, we observed significant disparity between the HPLC and the Nichols Advantage CPBA chemiluminescent assay for total 25(OH)D concentrations (Fig. 3A). Regression analysis yielded the following:

\[
y = 1.00x + 3.6 \mu g/L \quad (S_{y|x} = 32.7 \mu g/L; r = 0.6823; n = 54)
\]

Comparison of the results obtained with the HPLC method and the Nichols CPBA for samples from what appear to be 2 separate patient groups, one group with serum 25(OH)D concentrations composed predominantly of 25(OH)D₃ and the other group with predominantly 25(OH)D₂, are shown in panels B and C of Fig. 3. HPLC and Diasorin RIA [¹²⁵I-labeled 25(OH)D tracer] assay results for serum samples collected from a large group of college students are presented in Fig. 4. Nearly all samples contained only 25(OH)D₃.

**Discussion**

**Technical Considerations**

The analysis of VitD metabolites presents a unique challenge. The highly lipophilic compounds strongly associate with VitD-binding protein (VDBP), a bond that must be broken to release the metabolites for efficient liquid-liquid or solid-phase extraction. Moreover, endogenous lipids readily coextract with the metabolites and produce visibly “dirty” extracts that can foul the HPLC column and distort chromatographic peak shape. Clean-up steps are essential for a reliable, stable HPLC method (4). Light, especially direct natural sunlight, will rapidly degrade the internal standard and 25(OH)D metabolites. Moreover, care must be exercised to prevent instability of the internal standard during the evaporation step. Temperatures in excess of 35 °C and/or extended periods under a nitrogen stream after solvent evaporation can decrease recovery of the internal standard. Furthermore, recovery of the internal standard will differ with changes in volume of the serum tested and when diluted serum is used. Accordingly, the volumes of calibrators and controls extracted must be the same as the volume tested for patient samples. The smallest sample size for this method is 0.5 mL; the LLQ would then be 10 μg/L. Laurophenone was chosen as the internal standard because it separates from the 25(OH)D metabolites and other endogenous compounds. We evaluated a series of compounds that are structurally similar to the 25(OH)D metabolites but found no good candidates.

Several attempts have failed to standardize, or at least harmonize, 25(OH)D results from different laboratories, which suggests a complex problem as the root source of variability. A starting point would be the production of certified pure 25(OH)D₃ and 25(OH)D₂ substances in quantities that can be easily measured gravimetrically. Historically, the convention has been to assign a value to stock calibrator solutions of 25(OH)D₃ and 25(OH)D₂ through spectrophotometric analysis and calculations using molar absorptivity factors. Much would be gained with the availability of accurate calibrators, perhaps through the NIST. Equally important would be the availability of commercial control products containing both 25(OH)D₃ and 25(OH)D₂. Likewise, a serum product

![Fig. 4. Comparison of the proposed HPLC method with the Diasorin RIA.](image-url)
containing no endogenous 25(OH)D$_3$ and 25(OH)D$_2$ should be obtainable as a base material for preparing calibrators.

COMPARISION STUDIES
We obtained nearly identical results for 25(OH)D$_3$ and 25(OH)D$_3$ in patient samples with the HPLC and LC-MS/MS methods. LC-MS/MS, however, requires expensive instrumentation and considerable technical expertise, whereas the proposed HPLC method is less technically demanding and the costs are lower. In our hands, the Nichols Advantage CPBA method overestimated total 25(OH)D metabolite concentrations by an average of 58% when 25(OH)D$_3$ was the predominant form in serum and underestimated concentrations by an average of 27% when 25(OH)D$_2$ was the predominant form. The standard error of the residuals for each group was significant at 31.6 and 8.69 μg/L, respectively. These results are not surprising because the selectivity of VDBP is highly dependent on the extraction/purification techniques used before sample incubation with the reactive protein. Cross-reactivity with the VitD metabolites 24,25- and 24,26-dihydroxyvitamin D, potential cross-reactivity with 25(OH)-epi-D$_3$ and 25(OH)D sulfate, and deleterious interactions with serum matrix (lipids) could compromise accuracy. According to Shimada et al. (22), nearly equal amounts of 25(OH)D$_3$ sulfate and 25(OH)D$_3$ are usually present in patient sera. Data from several investigators (4, 23, 24) support our conclusion concerning the performance of the Nichols Advantage CPBA assay in clinical samples. Most interesting is the recent communication in Clinical Chemistry by a spokesperson for Nichols Company, demonstrating a reluctance to confirm that problems may exist with the assay (25).

Some VDBP-based assays forgo the traditional liquid-liquid or solid-phase sample clean-up for convenience and to minimize the need for manual intervention. Unfortunately, reports of inaccuracy have brought into question the reliability of the VDBP-based assays (26, 27).

In 1985, the first "valid” RIA for circulating 25(OH)D was introduced (28). A $^3$H-labeled 25(OH)D tracer was used initially but was replaced by an $^{125}$I-labeled 25(OH)D tracer in 1993. The Hollis RIA was subsequently offered by Diasorin, which later incorporated chemiluminescent detection on the Liaison platform. Both assays use the same antibody but when compared gave different results (29). Our data both confirm and contradict other investigators’ observations, which points to user reliance on the accuracy of the procedures used in the evaluation. Maunsell et al. (16) reported that VDBP-based assays forgo the traditional liquid-liquid or solid-phase sample clean-up for convenience and to minimize the need for manual intervention. Unfortunately, reports of inaccuracy have brought into question the reliability of the VDBP-based assays (26, 27).

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In our laboratory provides 25(OH)D testing 5 days a week and meets the needs of a medium-size university hospital. Daily testing is performed by 1 of 6 analysts on a rotating basis. Extractions are routinely performed during the day shift, and samples are analyzed overnight by an unattended HPLC to obtain results the next morning. Currently, we can process up to 50 samples per day. The assay has been in use for 10 months, and we typically assay an average of 600 samples each month. Moreover, 2 laboratories outside of our hospital have successfully replicated our method.

In summary, the variability among current commercial immunoassays and the inaccuracy of the Nichols Advantage CBPA in measuring 25(OH)D$_2$ and 25(OH)D$_3$ bring into question the benefit these assays have for patients. In light of these observations, validated LC-MS/MS and HPLC procedures would be the preferred techniques for measuring these 2 metabolites.

References