Transfer of 25-Hydroxyvitamin D2/D3 Analysis in Human Plasma to Core-Shell LC Technology to Improve Sample Throughput in a Clinical Laboratory

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This poster describes a sensitive, rugged and robust, high-throughput method for the analysis of 25-OH vitamin D2 and 25-OH vitamin D3 in serum. Following protein precipitation to eliminate proteins and disrupt serum protein binding of 25-OH vitamin D2 and 25-OH vitamin D3, the chromatographic separation of 25-OH vitamin D2 and 25-OH vitamin D3 is carried out in less than five minutes using a Kinetex® 2.6 µm C18 core-shell column, with total chromatographic cycle time of six minutes. This method provides rapid, sensitive, rugged, and robust LC/MS/MS analysis of Vitamin D levels in patient serum (LOD of 1 and 2 ng/mL for 25-OH D3 and 25-OH D2, respectively with CV of 4-7 %) and speeds the diagnosis by hospital and clinical laboratories of potential vitamin D deficiencies.
Vitamin D is recognized as an essential nutrient with its primary physiological function being to increase intestinal absorption of calcium and phosphate and to promote deposition of these minerals in newly formed bones. Deficiency and abnormal vitamin D levels result in impaired bone mineralization and lead to bone softening diseases – rickets in children and osteomalacia in adults. In addition, a large number of bone disorders and mineral metabolism defects have been associated with abnormal vitamin D levels, including nephritic syndrome, granulomatous diseases and hypocalcemia and secondary hyperparathyroidism that frequently complicates renal failure. As a result vitamin D testing has increased tremendously.1

Vitamin D is metabolized to 25-hydroxyvitamin D (25-OH D) in the liver. Total vitamin D is best determined by measuring total 25-OH D (D2 and D3) in serum since the half-life of 25-OH D is about three weeks with serum concentrations of 10 – 50 ng/mL. Vitamin D supplementation in both food and tablets comes in both the D2 and D3 forms, making it imperative to measure 25-OH D2 and 25-OH D3. While optimal serum concentrations of total 25-OH D are generally agreed to be ≥ 30 ng/mL, there is considerable discussion regarding the serum concentration of 25-OH D regarded as inadequate for bone and overall health but < 20 ng/mL is generally regarded as deficient. Serum concentrations > 100 ng/mL are generally regarded as potentially toxic.2

Vitamin D exhibits a high propensity for inherent endogenous serum protein binding and association. Vitamin D is typically not found free in serum samples, thereby posing a challenge for sensitive and reproducible HPLC analysis without appropriate sample preparation. Additionally, various serum sample matrix constituents are found to cause ion-suppression that reduces accuracy and reproducibility during patient sample analysis. This method provides rapid, sensitive, rugged and robust LC/MS/MS analysis of Vitamin D levels in patient serum (LOD of 1 and 2 ng/mL for 25-OH D3 and 25-OH D2, respectively with CV of 4-7 %) and speeds the diagnosis by hospital and clinical laboratories of potential vitamin D deficiencies.
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Sample Preparation

1. Add 350 µL of the precipitating reagent containing internal standard to a 1.5 mL centrifuge tube.
2. Pipette 100 µL of serum into the centrifuge tube.
4. Visually inspect each tube to ensure no unmixed sample remains in the bottom of the tube.
   a. It is critical that a homogeneous mixture is obtained.
   b. If unmixed sample remains at the bottom of the tube, dislodge by inverting and tapping, then re-vortex.
5. Centrifuge for 15 minutes at 13000 rpm.
6. Carefully transfer supernatant into sample vial without disturbing the pellet.

<table>
<thead>
<tr>
<th>Instrument</th>
<th>API 4000</th>
<th>4000 QTRAP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ionization</td>
<td>APCI</td>
<td>APCI</td>
</tr>
<tr>
<td>Scan Type</td>
<td>MRM</td>
<td>MRM</td>
</tr>
<tr>
<td>Polarity</td>
<td>Positive</td>
<td>Positive</td>
</tr>
<tr>
<td>Curtain Gas (CUR)</td>
<td>10.00</td>
<td>15.00</td>
</tr>
<tr>
<td>Nebulizer Current</td>
<td>5.00</td>
<td>5.00</td>
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<tr>
<td>Temperature (TEM)</td>
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<td>Gas 1 (GS1)</td>
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<td>Gas 2 (GS2)</td>
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<td>0.00</td>
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<td>Collision Gas (CAD)</td>
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<td>High</td>
</tr>
<tr>
<td>Entrance Potential (EP)</td>
<td>10.00</td>
<td>3.30</td>
</tr>
<tr>
<td>Interface Heater (ihe)</td>
<td>ON</td>
<td>ON</td>
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</table>
Analysis of 25-OH D2 and 25-OH D3 from serum necessitates the use of simple sample preparation procedures to remove potential matrix constituents, which will interfere with accurate and precise determination of 25-OH D in serum and reduce HPLC column lifetime. Vitamin D and the hydroxy D metabolites are relatively more hydrophobic than virtually all endogenous and exogenous compounds that are typically quantified in biological matrices. The propensity for vitamin D to inherently associate with serum proteins reduces the bioavailability of free vitamin D in serum. Therefore, it is necessary to disrupt this association for improved assay accuracy and precision. Protein precipitation is the easiest means of disrupting the serum protein association with hydrophobic analytes. In this method, protein precipitation is performed in 1.5 mL centrifuge tubes with the addition of 100 µL of serum sample to 350 µL of acetonitrile/methanol (95:5) containing the 25-OH D3-d6 internal standard. The low solubility of the endogenous proteins in acetonitrile results in their precipitation from the sample; mixing and centrifugation cause the precipitated protein to form a pellet at the bottom of the centrifuge tube and the supernatant is then analyzed. An alternative to the traditional protein precipitation approach involves the use of Strata® Impact™ protein precipitation plates, which contain an oleophobic membrane filter. Following addition of the serum sample to acetonitrile to facilitate protein precipitation, the sample passes through a cut-off filter to eliminate the precipitated protein from the sample.

MS/MS acquisition was performed in multiple reaction monitoring (MRM) mode, see Tables 1 and 2 for the MRM transitions monitored using the API 4000 and 4000 QTRAP, respectively. The use of MRM is important since the 25-OH D2 and 25-OH D3 are not separated chromatographically; however, the unique parent/daughter ion combination for each analyte allows for specificity and accurate determination of the concentration for each analyte in the sample. 25-OH D3-d6 was used as an internal standard while signal intensity of each 25-OH D analyte relative to the internal standard was used for determining the concentration of 25-OH D2 and 25-OH D3 in the sample. The calibration curves were linear over the range 0 to > 500 ng/mL, with observed limits of detection (LOD) of 1 and 2 ng/mL for 25-OH D3 and 25-OH D2, respectively. The reproducibility of this assay was very good with CV of 4-7%.

Both the HPLC and UHPLC conditions using a fully porous 5 µm C18 column (50 x 2.0 mm) and the Kinetex® 2.6 µm C18 column (50 x 4.6 mm), respectively, allow for sufficient retention of 25-OH D2 and 25-OH D3, further minimizing the potential for interference and ion-suppression from any weakly retained impurities (Figures 1 and 2). Using the five µm C18 column, elution of 25-OH D2 and 25-OH D3 occurred in just under five minutes, with overall chromatographic run time of eight minutes – including column re-equilibration. The separation using the Kinetex core-shell C18 column is similar. However, 25-OH D2 and 25-OH D3 elute in less than four minutes. This allows the overall chromatographic run time, including column re-equilibration, to be reduced to six minutes. The shorter analysis time using the Kinetex core-shell column is a significant benefit for laboratories analyzing a large number of samples in a high-throughput sample environment – the reduction in overall chromatographic run time translates into a 25 % increase in sample throughput and corresponding reduction in solvent usage. In addition to the faster chromatographic separation, the peak intensities are significantly larger on the core-shell Kinetex column resulting in improved sensitivity and lower quantitation limits. However, the most important benefit is the improved efficiency and resolution provided by the core-shell column.

With the fully porous 5 µm C18 column, an endogenous compound (D3 Cholesterol) present in patient samples was found to co-elute with the 25-OH D3 peak, impacting accurate quantitation and increasing the need to re-analyze a large percentage of patient samples (Figure 3). The presence of the endogenous compound in patient samples was resolved from 25-OH D3 and found to elute outside the quantitation window on the Kinetex core-shell column (Figure 4), resulting in increased analytical accuracy.
<table>
<thead>
<tr>
<th>Analyte</th>
<th>MRM Pair (Q1/Q3)</th>
<th>Dwell Time (sec)</th>
<th>DP</th>
<th>CE</th>
<th>CXP</th>
</tr>
</thead>
<tbody>
<tr>
<td>25-OH D2</td>
<td>395.3 / 209.3</td>
<td>200</td>
<td>66.0</td>
<td>20.0</td>
<td>6.0</td>
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<tr>
<td>25-OH D3</td>
<td>383.2 / 257.2</td>
<td>200</td>
<td>66.0</td>
<td>31.0</td>
<td>13.0</td>
</tr>
<tr>
<td>25-OH D3-d₆</td>
<td>389.3 / 263.3</td>
<td>200</td>
<td>82.0</td>
<td>30.0</td>
<td>15.0</td>
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Note: Ion source and mass dependent parameters may require minor modification on any individual system to achieve the optimal sensitivity and performance.
Table 2. **4000 QTRAP**

<table>
<thead>
<tr>
<th>Analyte</th>
<th>MRM Pair (Q1/Q3)</th>
<th>Dwell Time (sec)</th>
<th>DP</th>
<th>CE</th>
<th>CXP</th>
</tr>
</thead>
<tbody>
<tr>
<td>25-OH D2</td>
<td>395.3 / 209.3</td>
<td>200</td>
<td>65.0</td>
<td>20.0</td>
<td>6.4</td>
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<tr>
<td>25-OH D3</td>
<td>383.2 / 257.2</td>
<td>200</td>
<td>70.0</td>
<td>34.0</td>
<td>16.8</td>
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<tr>
<td>25-OH D3-d₆</td>
<td>389.3 / 263.3</td>
<td>200</td>
<td>88.0</td>
<td>23.0</td>
<td>19.0</td>
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</table>

Note: Ion source and mass dependent parameters may require minor modification on any individual system to achieve the optimal sensitivity and performance.
**Figure 1. Fully Porous 5 µm C18 50 x 2.0 mm Standard Chromatogram**

Column: Luna® 5 µm C18(2)
Dimensions: 50 x 2.0 mm
Part No.: 00B-4252-B0
Mobile Phase: A: 0.05 % Formic acid
B: 5 mM Ammonium acetate with 0.1 % Formic acid in Methanol
Gradient: Time (sec) B %
0 8
5 8
205 100
290 100
360 8
Flow Rate: 0.9 mL/min
Temperature: 35 ºC
Detection: Mass Spectrometer (MS)
Sample:
1. 25-hydroxyvitamin D3 (25-OH D3) MRM: 383.3 / 257.2
2. 25-hydroxyvitamin D3-d6 (25-OH D3-d6) MRM: 389.3 / 263.3
3. 25-hydroxyvitamin D2 (25-OH D2) MRM: 395.3 / 209.3
**Figure 2. Kinetex 2.6 µm C18 50 x 4.6 mm Standard Chromatogram**

**Column:** Kinetex 2.6 µm C18  
**Dimensions:** 50 x 4.6 mm  
**Part No.:** 00B-4462-E0  
**Mobile Phase:**  
A: 0.05 % Formic acid  
B: 5 mM Ammonium acetate with 0.1 % Formic acid in Methanol  
**Gradient:**  
<table>
<thead>
<tr>
<th>Time (sec)</th>
<th>B %</th>
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<tbody>
<tr>
<td>0</td>
<td>8</td>
</tr>
<tr>
<td>5</td>
<td>8</td>
</tr>
<tr>
<td>205</td>
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<td>290</td>
<td>100</td>
</tr>
<tr>
<td>360</td>
<td>8</td>
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**Flow Rate:** 1 mL/min  
**Temperature:** 35 ºC  
**Detection:** Mass Spectrometer (MS)  
**Sample:**  
1. 25-hydroxyvitamin D3 (25-OH D3) MRM: 383.2 / 257.2  
2. 25-hydroxyvitamin D3-d6 (25-OH D3-d6) MRM: 389.3 / 263.3  
3. 25-hydroxyvitamin D2 (25-OH D2) MRM: 395.2 / 209.3
Figure 3. Fully Porous 5 µm C18 50 x 2.0 mm Sample Chromatogram

Column: Luna 5 µm C18(2)
Dimensions: 50 x 2.0 mm
Part No.: 00B-4252-B0
Mobile Phase: A: 0.05 % Formic acid
B: 5 mM Ammonium acetate with 0.1 % Formic acid in Methanol
Gradient: Time (sec) B %
0 8
5 8
205 100
290 100
360 8
Flow Rate: 1 mL/min
Temperature: 35 ºC
Detection: Mass Spectrometer (MS)
Sample:
1. 25-hydroxyvitamin D3 (25-OH D3) MRM: 383.2 / 257.2
2. 25-hydroxyvitamin D3-d6 (25-OH D3-d6) MRM: 389.3 / 263.3
3. 25-hydroxyvitamin D2 (25-OH D2) MRM: 395.2 / 209.3
**Figure 4. Kinetex 2.6 µm C18 50 x 4.6 mm Sample Chromatogram**

**Column:** Kinetex 2.6 µm C18  
**Dimensions:** 50 x 4.6 mm  
**Part No.:** 00B-4462-E0  
**Mobile Phase:**  
- A: 0.05 % Formic acid  
- B: 5 mM Ammonium acetate with 0.1 % Formic acid in Methanol  
**Gradient:**  
<table>
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<tr>
<th>Time (sec)</th>
<th>B %</th>
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<td>290</td>
<td>100</td>
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<tr>
<td>360</td>
<td>8</td>
</tr>
</tbody>
</table>
**Flow Rate:** 1 mL/min  
**Temperature:** 35 ºC  
**Detection:** Mass Spectrometer (MS)  
**Sample:**  
1. 25-hydroxyvitamin D3 (25-OH D3) MRM: 383.2 / 257.2  
2. 25-hydroxyvitamin D3-d6 (25-OH D3-d6) MRM: 389.3 / 263.3  
3. 25-hydroxyvitamin D2 (25-OH D2) MRM: 395.2 / 209.3
Conclusions

This analytical method allows for the rapid and accurate determination of vitamin D levels in patient serum samples and speeds the diagnosis of potential vitamin D deficiencies indicative of specific disease states quickly and with a high degree of precision.

Protein precipitation is used for sample preparation of patient serum samples, effectively disrupting the serum protein association with 25-OH D2 and 25-OH D3 and providing sufficient sample cleanup prior to LC/MS/MS analysis. Strata Impact protein precipitation plates would be an effective alternative for sample preparation in a high-throughput clinical laboratory environment.

The Kinetex core-shell technology allows for faster chromatographic analysis of patient samples and increased signal intensity for improved sensitivity. In addition, the increase in chromatographic resolution from an endogenous compound present in patient serum samples provides improved accuracy and reproducibility, and a decrease in sample re-analysis.