Rapid Separation of 25-OH-Vitamin D3 and 3-Epi-25-OH-Vitamin D3 in Human Serum using Tandem Mass Spectrometry Detection

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Faulty vitamin D metabolism in children less than 12 months of age can lead to formation of the inactive 3-epi-25 monohydroxy form. The resolution of 3-epimer from the active monohydroxy form by tandem mass spectrometry is not possible due to mostly identical fragmentation pattern of the two species. As a result, the two isomers should be separated chromatographically. The method described here resolves the critical pair within a short run time.

Serum/plasma samples were treated with acetonitrile to precipitate the protein, followed by centrifugation. A small volume of the supernatant was injected on the LC column. The chromatographic separation is carried out by a high efficiency media that allowed for separation of the monohydroxy vitamin D3 isomers as well as separation of the 3-epi-25 monohydroxy epimer. A typical methanol and formic acid mobile phase combination starting with high organic concentration is used. The column is maintained at ambient temperature, ~22 °C. The signal detection is carried out by a triple quadrupole mass spectrometer operating in multiple reactions monitoring (MRM) function. An atmospheric pressure ionization source operating in positive polarity and using high purity nitrogen gas produced the [M+H-H2O]+ precursor ions. The LOD for both 25-OH-Vit D3 and its 3-epimer were similar at 2.5 ng/mL.

The method prescribed here provides excellent resolution of the monohydroxy vitamin D3 isomers within a short run time.
In recent years, vitamin D (Ergocalciferol, D2 and Cholecalciferol, D3) has been subject to increasing investigation for a range of potentially beneficial health effects. The measurement of Vitamin D metabolites, 25-hydroxy (25-OH) and 1α, 25-DiOH vitamin D (Vit D), is used as a marker to determine vitamin D deficiency. Isomerization of 25-OH-Vit D produces 3-epi Vit D3 (conversion of α-OH to β-OH), a diasteromeric form. The presence of the epimer was first reported in 2006 by Singh et al. In infants, a significant portion of the 25-OH Vit D may be present as the epimeric form. Thus, in order to determine the accurate vitamin D status of such patients, it is necessary to be able to distinguish between the two diastereomeric forms.

Historically, analysis of Vit D and its metabolites has been performed via immunoassays. However, there is some question as to the ability of immunoassays to discriminate between 25-OH-D3 and its epimer. Thus, the development of an LC/MS/MS analysis that can distinguish the 25-OH-Vit D metabolite from its epimeric form is greatly desired.
Instrumentation and Conditions

LC System:
Agilent® 1260 UPLC System with binary LC pumps

HPLC conditions: As specified on the chromatogram

MS System:
AB SCIEX API 5000™ operating under Pos polarity APCI
MS Parameters:

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
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<tbody>
<tr>
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<tr>
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<td>Temperature (TEM)</td>
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<tr>
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<td>Q1, Da</td>
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<td>---------------------</td>
<td>--------</td>
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<tr>
<td>OH-Vit D2</td>
<td>395.3</td>
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<tr>
<td>OH-Vit D3/Epi-D3</td>
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</tr>
<tr>
<td>Int Std (OH-D3-2H₃)</td>
<td>386.2</td>
</tr>
<tr>
<td>OH-Vit D3 (Sec Trans)</td>
<td>383.2</td>
</tr>
<tr>
<td>OH-Vit D2 (Sec Trans)</td>
<td>395.3</td>
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Chromatographic Media

2.6 μm Core-Shell Particle

- The final, optimized LC/MS/MS method for the separation and analysis of 25-OH-Vit D and its epimer was performed using a core-shell column - Kinetex® 2.6 μm PFP.
- The core-shell Kinetex particle consists of a solid inner core surrounded by a layer of porous silica material.
- This unique core-shell structure can provide exceptionally high efficiency at relatively modest backpressure (compatible with a conventional HPLC system).

• 0.35 μm Porous Shell
• 1.9 μm Solid Core

Performance equivalent to or better than fully porous

Can be used on conventional HPLC systems and UH-
Although the majority of reversed phase HPLC methods are developed using C18 bonded phases, the C18 stationary phase chemistry lacks the ability to adequately resolve the 25-OH-Vit D3 from its epimer.
A phenyl-based stationary phase bonded to conventional fully-porous silica (Synergi™ Polar-RP) was able to adequately resolve 25-OH-Vit D2 from 25-OH-Vit D2, but it did not display any resolution of the 25-OH-Vit D3 epimeric form.
Using the core-shell Kinetex PFP column in a water/acetonitrile/formic acid mobile phase, it is possible to separate 25-OH-D3 from its epimer, and also to separate out the 25-OH-D2 in a run time of about 10 minutes.
• By switching to a mobile phase containing methanol rather than acetonitrile, we can take advantage of the unique PFP selectivity to separate 25-OH-D2 from 25-OH-D3 and also to fully-resolve the epimeric 25-OH-D3 metabolite with a total analysis time less than 5 minutes.
• Commercially-available human serum contains relatively high levels of both 25-OH-D3 and its epimer, making it unsuitable for use in making a calibration curve.

• Because of this, we used double charcoal-stripped human serum, which was found to have significantly lower levels of these components.
Sample Preparation

• A protein precipitation method was devised to establish a calibration curve from 2.5 to 100 ng/mL.
• Commercially available serum could not be used due to its high contents of the OH-Vit D3 AND 3-epi forms (Figure 5).
• Double charcoal-stripped human serum was tested and found to have lower than 2.5 ng/mL concentration of OH-D2/D3.
• Sample preparation was carried out with the below procedure:
  — 30 µL Int Std (OH-D3-2H3) and 200 µL sample was treated with 400 µL precipitation reagent (5:2:1 Methanol/Acetonitrile/Zinc Sulfate) and vortexed briefly, 4-5 sec
  — The mixture was centrifuged at 14000 rpm for 7 minute
  — The supernatant was decanted into an autosampler vial and placed in the autosampler
• A linear fit with 1/x weighting factor was used for both analytes and showed an excellent calibration fit (Figures 6-7.)
Figure 6. **OH-Vit D3 calibration curve from 2.5 to 100 ng/mL**

- Calibration curve for OH-Vit D3 from 2.5 to 100 ng/mL, $r=0.9984$
**Figure 7.** OH-Vit D2 calibration curve from 2.5 to 100 ng/mL

* Calibration curve for OH-Vit D2 from 2.5 to 100 ng/mL, r=0.9994
We have developed an assay using the Kinetex 2.6 μm PFP column that can accurately quantitate 25-OH-Vit D3 in the presence of its epimeric form using a simple water/methanol/formic acid mobile phase. This assay can also be used to quantitate 25-OH-D2, 25-OH-D3, and the 25-OH-D3 epimer with a total analysis time of less than 5 minutes.

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