Rapid Separation of 25-OH-vitamin D3 and 3-epi-25-OH-vitamin D3 in Human Serum Under RP-LC Conditions and 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 the 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.

Introduction

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 1⟨, 25-DiOH vitamin D (Vit D), is used as marker to determine vitamin D deficiency. Isomerization of 25-OH-Vit D produces 3-epi Vit D3 (conversion of ⟨-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-OHVit 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-OHVit D metabolite from its epimeric form is greatly desired.

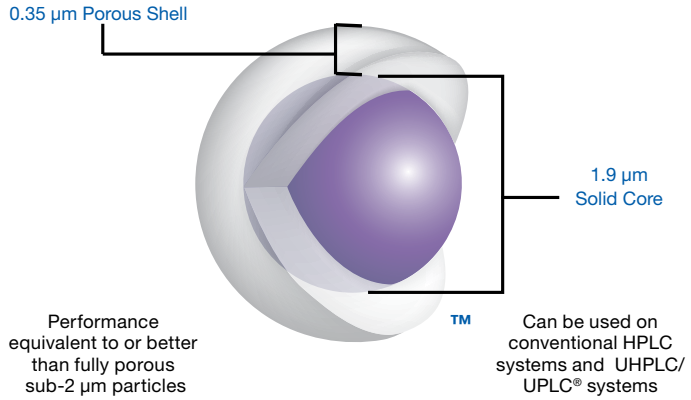
Instrumentation and Conditions

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: Gas 1 (GS1) 40
Curtain Gas (CUR) 25
Temperature (TEM) 350 °C
Nebulizer Current (NC) 5 µA
Collision Gas (CAD) 8
DP 100 V
Entrance Potential (EP) 10 V
CXP 10 V
Dwell 150 msec

Table 1.
MRM Transitions Table

Compound ID	Q1, Da	Q3, Da	CE, V
OH-Vit D2	395.3	209.3	30
OH-Vit D3/Epi-D3	383.2	257.2	25
Int Std (OH-D3-$^2H_3$)	386.2	257.2	25
OH-Vit D3 (Sec Trans)	383.2	229.1	30
OH-Vit D2 (Sec Trans)	395.3	269.2	22

2.6 µm Kinetex® 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.

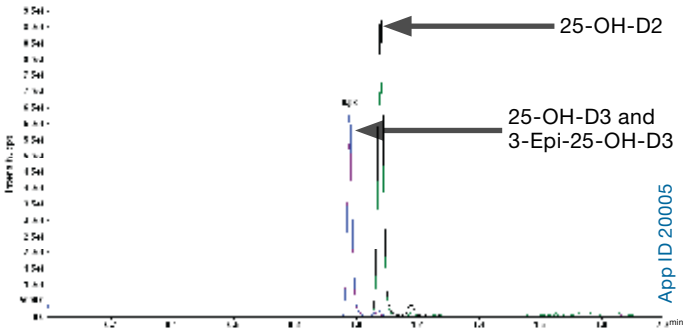
- The core-shell Kinetex particle consists of a solid inner core surrounded by a layer of porous silica material.
- This unique core-shell structure can provide exceptionally high efficiency at relatively modest backpressure (compatible with a conventional HPLC system).

Results and Discussion

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.

Figure 1.

Separation using a fully porous silica bonded with C18 chemistry



Column: Kinetex 2.6 µm C18
Dimensions: 30 x 3.0 mm
Part No.: 00A-4462-Y0
Mobile Phase: A. 0.1 % Formic Acid in DI water
B. 0.1% Formic Acid in Acetonitrile

Gradient:

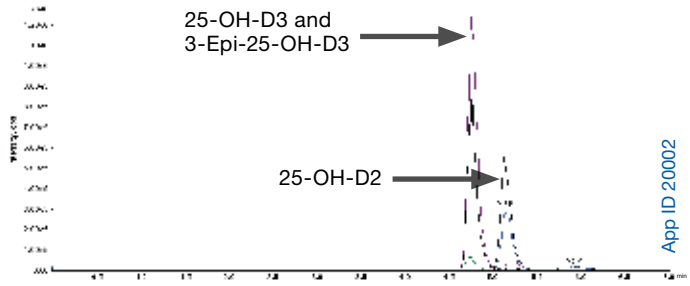
Time (min)	% B
0	55
0.3	80
1	80
1.1	95
1.5	95
1.51	55
2	55

Flow Rate: 400 µL
Temperature: Ambient
Detection: Tandem Mass Spec (MS-MS)
Instrument: API 5000

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.

Figure 2.

Separation using a fully porous silica bonded with phenyl chemistry



Column: Synergi™ 2.5 µm Polar-RP
Dimensions: 100 x 2.0 mm
Part No.: 00D-4371-B0
Mobile Phase: A. 0.1 % Formic Acid in DI water
B. 0.1% Formic Acid in Methanol

Gradient:

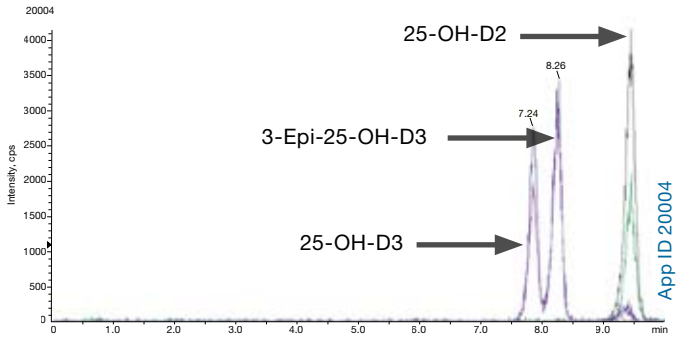
Time (min)	% B
0	75
2	80
3.8	80
3.81	75
6	75

Flow Rate: 400 µL
Temperature: Ambient
Detection: Tandem Mass Spec (MS-MS) (ambient)
Instrument: API 5000

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.

Figure 3.

Separation using the Kinetex PFP and Acetonitrile

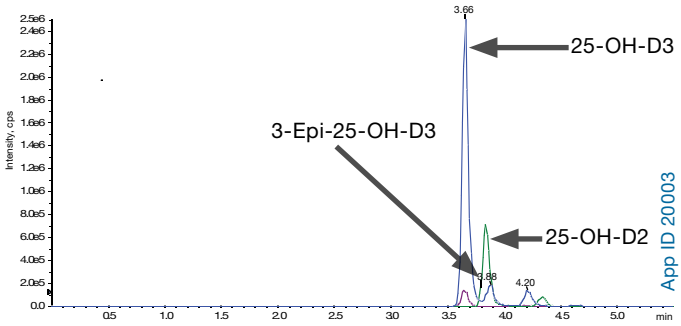


Column: Kinetex 2.6 µm PFP
Dimensions: 100 x 2.1mm
Part No.: 00D-4477-AN
Mobile Phase: A.0.1% Formic Acid in DI water
B 0.1% Formic Acid in Acetonitrile (55:45)
Flow Rate: 400 µL
Temperature: Ambient
Detection: Tandem Mass Spec (MS-MS)
(ambient)

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.

Figure 4.

Separation using the Kinetex PFP and Methanol



Column: Kinetex 2.6 µm PFP
Dimensions: 100 x 2.1 mm
Part No.: 00D-4477-AN
Mobile Phase: A. 0.1 % Formic Acid in DI water
B. 0.1% Formic Acid in Methanol

Gradient:

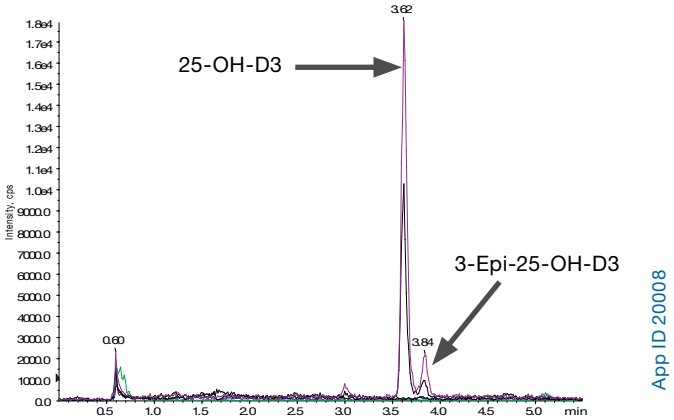
Time (min)	% B
0	75
2	80
3.8	80
3.81	75
6	75

Flow Rate: 400 µL
Temperature: Ambient
Detection: Tandem Mass Spec (MS-MS)
Instrument: API 5000

APPLICATIONS

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.

Figure 5.
Chromatogram of a commercially available human serum



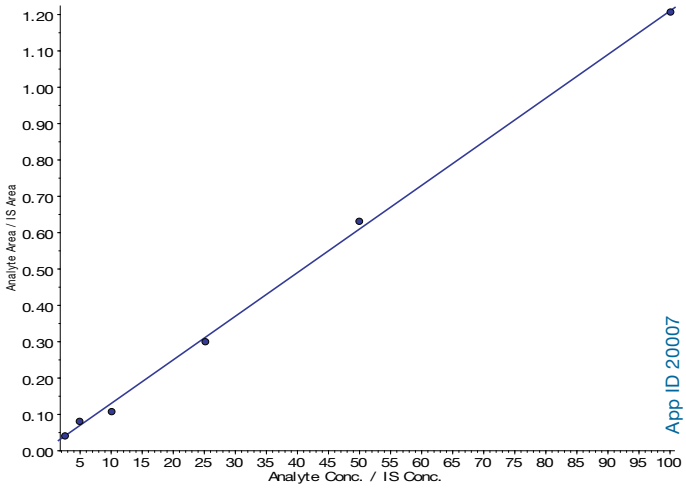
Column: Kinetex 2.6 µm PFP
Dimensions: 100 x 2.1mm
Part No.: 00D-4477-AN
Mobile Phase: A.0.1% Formic Acid in DI water
B 0.1% Formic Acid in Acetonitrile (55:45)
Flow Rate: 400 µL
Temperature: Ambient
Detection: Tandem Mass Spec (MS-MS) (ambient)

Sample Preparation

A protein precipitation method was devised to establish a calibration curve from 2.5 to 100 ng/mL.

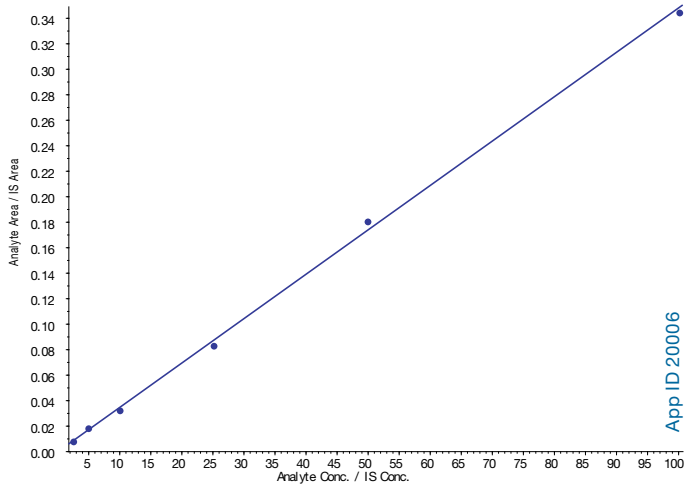
- Commercially available serum could not be used due to its high contents of the OH-Vit D3 AND 3-epi forms (**Figure 5**).
- Double charcoal-stripped human serum was tested and found to have lower than 2.5 ng/mL concentration of OH-D2/D3.
- Sample preparation was carried out with the below procedure:
  - —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 to100 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 to100 ng/mL



- Calibration curve for OH-Vit D2 from 2.5 to 100 ng/mL, r=0.9994

Conclusion

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-OHD3 epimer with a total analysis time of less than 5 minutes.

References

1. Singh et al, *J Clin Endocrinol Metab* **2006**; 91:3055–61
2. Hoofnagle et al, *Clinica Chimica Acta* **2012**; 413:203–206
2. Schlelcher et al, *Clinica Chimica Acta* **2012**; 412:1549–1599

APPLICATIONS

Ordering Information
Kinetex® 2.6 µm MidBore™ Columns (mm)

	30 x 3.0	50 x 3.0	75 x 3.0	100 x 3.0	150 x 3.0	SecurityGuard™ ULTRA Cartridges* 3/pk
XB-C18	00A-4496-Y0	00B-4496-Y0	00C-4496-Y0	00D-4496-Y0	00F-4496-Y0	AJ0-8775
C18	00A-4462-Y0	00B-4462-Y0	00C-4462-Y0	00D-4462-Y0	00F-4462-Y0	AJ0-8775
C8	00A-4497-Y0	00B-4497-Y0	00C-4497-Y0	00D-4497-Y0	00F-4497-Y0	AJ0-8777
PFP	00A-4477-Y0	00B-4477-Y0	00C-4477-Y0	00D-4477-Y0	00F-4477-Y0	AJ0-8780
HILIC	00A-4461-Y0	—	—	—	00F-4461-Y0	AJ0-8779
Phenyl-Hexyl	—	—	—	—	—	AJ0-8781

for 3.0 mm ID

Kinetex 2.6 µm Minibore Columns (mm)

	30 x 2.1	50 x 2.1	75 x 2.1	100 x 2.1	150 x 2.1	SecurityGuard ULTRA Cartridges* 3/pk
XB-C18	00A-4496-AN	00B-4496-AN	00C-4496-AN	00D-4496-AN	00F-4496-AN	AJ0-8782
C18	00A-4462-AN	00B-4462-AN	00C-4462-AN	00D-4462-AN	00F-4462-AN	AJ0-8782
C8	00A-4497-AN	00B-4497-AN	00C-4497-AN	00D-4497-AN	00F-4497-AN	AJ0-8784
PFP	00A-4477-AN	00B-4477-AN	00C-4477-AN	00D-4477-AN	00F-4477-AN	AJ0-8787
HILIC	00A-4461-AN	00B-4461-AN	00C-4461-AN	00D-4461-AN	00F-4461-AN	AJ0-8786
Phenyl-Hexyl	—	00B-4495-AN	—	00D-4495-AN	—	AJ0-8788

for 2.1 mm ID

Go to www.phenomenex.com to find more information on the Kinetex 1.7 µm core-shell particle and other Kinetex column dimensions like the 4.6 mm ID.

*SecurityGuard ULTRA cartridges require holder, Part No. AJ0-9000

Ordering Information
2.5 µm High Speed Technology (HST) Columns (mm)

Phases	30 x 2.0	50 x 2.0	100 x 2.0	50 x 3.0	100 x 3.0	50 x 4.6
Max-RP	00A-4372-B0	00B-4372-B0	00D-4372-B0	00B-4372-Y0	00D-4372-Y0	00B-4372-E0
Hydro-RP	00A-4387-B0	00B-4387-B0	00D-4387-B0	00B-4387-Y0	00D-4387-Y0	00B-4387-E0
Polar-RP	00A-4371-B0	00B-4371-B0	00D-4371-B0	00B-4371-Y0	00D-4371-Y0	00B-4371-E0
Fusion-RP	00A-4423-B0	00B-4423-B0	00D-4423-B0	00B-4423-Y0	00D-4423-Y0	00B-4423-E0

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