Ultra-fast LC/MS/MS Analysis of 25-OH Vitamin D2 and D3 from Serum

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This technical note 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 5 minutes using a Kinetex® 2.6 µm C18 core-shell column, with total chromatographic cycle time of 6 minutes. This method provides rapid, sensitive, rugged and robust LC/MS/MS analysis of Vitamin D levels in serum (LOD of 1 and 2 ng/mL for 25-OH D3 and 25-OH D2, respectively with CV of 4-7%).

Introduction

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 sample analysis. This method provides rapid, sensitive, rugged and robust LC/MS/MS analysis of Vitamin D levels in serum (LOD of 1 and 2 ng/mL for 25-OH D3 and 25-OH D2, respectively with CV of 4-7%).

Experimental

Reagents and Chemicals

25-Hydroxyvitamin D3 and 25-hydroxyvitamin D3-d6 were obtained from Medical Isotopes (Pelham, NH) and 25-hydroxyvitamin D2 was obtained from Sigma-Aldrich® (Milwaukee, WI). HPLC grade water (Milli-Q, Millipore, Billerica, MA) was used to prepare HPLC mobile phase and for sample preparation). Methanol and acetonitrile were obtained from Honeywell, Burdick & Jackson (Muskegon, MI).

Stock internal standard solution was prepared by dissolving the contents of a 5 mg vial of 25-hydroxyvitamin D3-d6 in 5.0 mL of ethanol. The precipitation reagent was prepared by adding 10 µL of internal standard stock solution to 60 mL of (95:5) acetonitrile/methanol in a 100 mL volumetric flask and diluting to volume with acetonitrile/methanol (95:5).

LC/MS/MS was performed using a HPLC system equipped with binary pump, autosampler, and column oven interfaced with an AB SCIEX™ 4000 QTRAP® or with an API 4000™ triple quadrupole. The ionization source was atmospheric pressure chemical ionization (APCI) run in positive ion mode.

Sample Preparation

1. To a 1.5 mL centrifuge tube add 350 µL of the precipitating reagent containing internal standard.
2. Pipette 100 µL of serum into the centrifuge tube.
3. Vortex for 20-30 seconds.
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.

MS/MS Conditions

Instrument	API 4000™	4000 QTRAP®
Ionization	APCI	APCI
Scan Type	MRM	MRM
Polarity	Positive	Positive
Curtain Gas (CUR)	10.00	15.00
Nebulizer Current	5.00	5.00
Temperature (TEM)	450.00	450.00
Gas 1 (GS1)	75.00	55.00
Gas 2 (GS2)	0.00	0.00
Collision Gas (CAD)	6.00	High
Entrance Potential (EP)	10.00	3.30
Interface Heater (ihe)	ON	ON

Results and Discussion

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.

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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 acetonitrile to the serum sample 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 **Table 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 5 µm C18 column, elution of 25-OH D2 and 25-OH D3 occurred in just under 5 minutes, with overall chromatographic run time of 8 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 4 minutes. This allows the overall chromatographic run time, including column re-equilibration, to be reduced to 6 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 having as the most important benefit 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 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 samples **(Figure 3)**. The presence of the endogenous compound in 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 1.
API 4000™ Triple Quadrupole

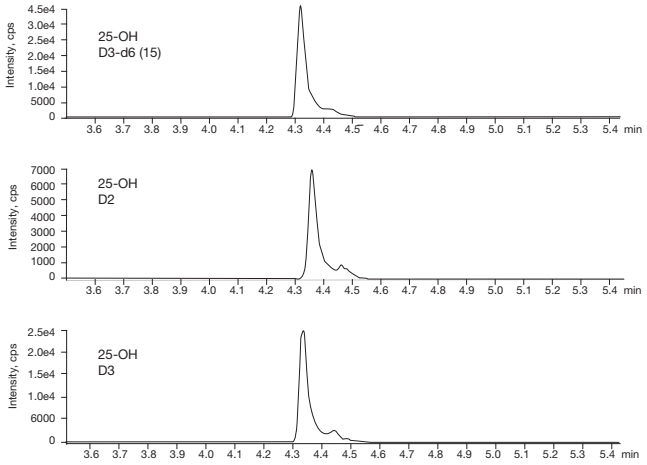
Analyte	MRM Pair (Q1/Q3)	Dwell Time (sec)	DP	CE	CXP
25-OH D2	395.3 / 209.3	200	66.0	20.0	6.0
25-OH D3	383.2 / 257.2	200	66.0	31.0	13.0
25-OH D3-d_6	389.3 / 263.3	200	82.0	30.0	15.0

Table 2.
4000 QTRAP®

Analyte	MRM Pair (Q1/Q3)	Dwell Time (sec)	DP	CE	CXP
25-OH D2	395.3 / 209.3	200	65.0	20.0	6.4
25-OH D3	383.2 / 257.2	200	70.0	34.0	16.8
25-OH D3-d_6	389.3 / 263.3	200	88.0	23.0	19.0

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



App ID 19315

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

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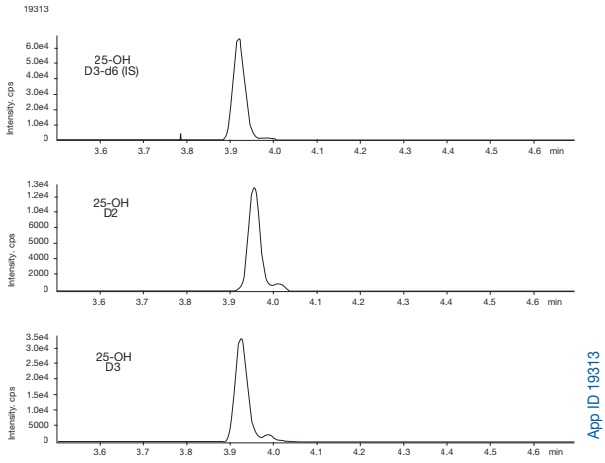
Figure 2.
Kinetex® 2.6 µm C18 50 x 4.6 mm Standard Chromatogram

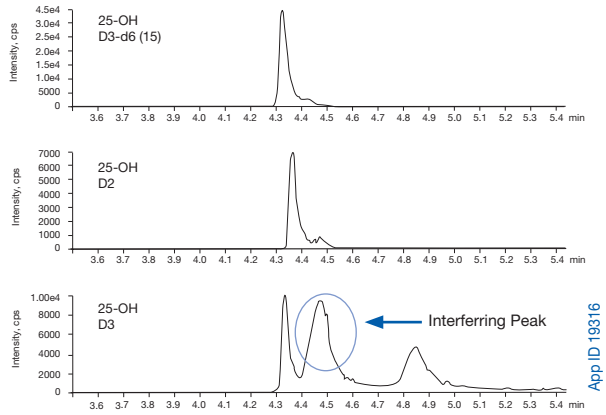


Column: Kinetex 2.6 µm C18
Dimensions: 50 x 4.6 mm
Part No.: 00B-4462-E0
Mobile Phase: A: 0.05 % Formic acid
B: 5 mM Ammonium acetate with 0.1 % Formic acid in methanol

Gradient:	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 3.
Fully Porous 5 µm C18 50 x 2.0 mm Sample Chromatogram

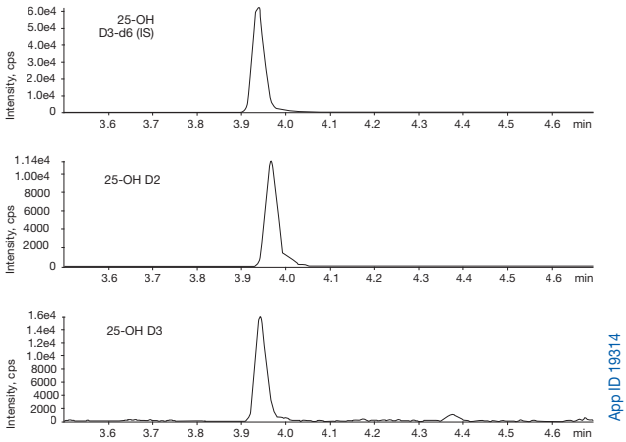


Column: Luna 5 µm C18(2)
Dimensions: 50 x 2.0 mm
Part No.: 00B-4252-B0
Mobile Phase: A: 0.05 % Formic acid
B: 5 mM Ammonium acetate with 0.1 % Formic acid in methanol

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:	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

Conclusions

This analytical method allows for the rapid and accurate determination of Vitamin D levels in serum samples.

Protein precipitation is used for sample preparation of 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 samples and increased signal intensity for improved sensitivity. In addition, the increase in chromatographic resolution from an endogenous compound present in serum samples provides improved accuracy and reproducibility, and a decrease in sample re-analysis.

References

1. Dietary Supplement Fact Sheet: Vitamin D; Office of Dietary Supplements, National Institutes of Health (http://ods.od.nih.gov/factsheets/vitamind.asp).
2. Institute of Medicine, Food and Nutrition Board. *Dietary Reference Intakes*: *Calcium, Phosphorus, Magnesium, Vitamin D, and Fluoride*. Washington, DC: National Academy Press, 1997.

APPLICATIONS

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Ordering Information

Strata® Impact™ Precipitation Plates

Part No.	Description	Unit/Box
CE0-7565	Strata Impact Protein Precipitation, Square Well, Filter Plate, 2 mL	2
CE0-8201	Kit consists of: Strata Impact Protein Precipitation Plate (2 ea); Collection Plate 2 mL (2 ea); Sealing Mat, Santoprene™ (AH0-8199) (2 ea)	ea

Kinetex® 2.6 µm Analytical Columns (mm)

	30 x 4.6	50 x 4.6	75 x 4.6	100 x 4.6	150 x 4.6	KrudKatcher™ Ultra In-Line Filter* /3pk
XB-C18	—	00B-4496-E0	00C-4496-E0	00D-4496-E0	00F-4496-E0	AF0-8497
C18	00A-4462-E0	00B-4462-E0	00C-4462-E0	00D-4462-E0	00F-4462-E0	AF0-8497
C8	—	00B-4497-E0	00C-4497-E0	00D-4497-E0	00F-4497-E0	AF0-8497
PFP	00A-4477-E0	00B-4477-E0	00C-4477-E0	00D-4477-E0	00F-4477-E0	AF0-8497
HILIC	—	00B-4461-E0	00C-4461-E0	00D-4461-E0	00F-4461-E0	AF0-8497

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	KrudKatcher Ultra In-Line Filter* /3pk
XB-C18	—	00B-4496-Y0	—	00D-4496-Y0	—	AF0-8497
C18	00A-4462-Y0	00B-4462-Y0	00C-4462-Y0	00D-4462-Y0	00F-4462-Y0	AF0-8497
C8	—	00B-4497-Y0	—	00D-4497-Y0	—	AF0-8497
PFP	00A-4477-Y0	00B-4477-Y0	00C-4477-Y0	00D-4477-Y0	00F-4477-Y0	AF0-8497
HILIC	—	—	—	—	00F-4461-Y0	AF0-8497

Kinetex 2.6 µm Minibore Columns (mm)

	30 x 2.1	50 x 2.1	100 x 2.1	150 x 2.1	KrudKatcher Ultra In-Line Filter* /3pk
XB-C18	00A-4496-AN	00B-4496-AN	00D-4496-AN	—	AF0-8497
C18	00A-4462-AN	00B-4462-AN	00D-4462-AN	00F-4462-AN	AF0-8497
C8	—	00B-4497-AN	00D-4497-AN	—	AF0-8497
PFP	00A-4477-AN	00B-4477-AN	00D-4477-AN	00F-4477-AN	AF0-8497
HILIC	—	00B-4461-AN	00D-4461-AN	00F-4461-AN	AF0-8497

Kinetex 1.7 µm Minibore Columns (mm)

	50 x 2.1	100 x 2.1	150 x 2.1	KrudKatcher Ultra In-Line Filter* /3pk
XB-C18	00B-4498-AN	00D-4498-AN	—	AF0-8497
C18	00B-4475-AN	00D-4475-AN	00F-4475-AN	AF0-8497
C8	00B-4499-AN	00D-4499-AN	—	AF0-8497
PFP	00B-4476-AN	00D-4476-AN	00F-4476-AN	AF0-8497
HILIC	00B-4474-AN	—	—	AF0-8497

*KrudKatcher Ultra requires $^{5}/_{16}$ in. wrench. Wrench not provided.

UHPLC / HPLC Sure-Lok™ High Pressure PEEK™ Male Nut Fittings

Part No.	Description	Unit
AQ0-8503	Sure-Lok High Pressure PEEK 1-Pc Nut 10-32, For $^{1}/_{16}$ in. Tubing, 12,000 psi (827 bar)	10/pk
AQ0-8530	Sure-Lok Fitting Tightening Tool, Aluminum	ea

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