SPE and LC-MS/MS Method for the Determination of 25-Hydroxyvitamin D2 and 25-Hydroxyvitamin D3 from Human Plasma

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Key Words
Solid phase extraction, SPE, SOLA HRP, LC-MS/MS, Syncronis C18, 25-hydroxyvitamin D2, 25-hydroxyvitamin D3

Abstract
A liquid chromatography - tandem mass spectrometry method for 25-hydroxyvitamin D2 and 25-hydroxyvitamin D3 from human plasma has been developed. Sample preparation was fast and efficient using a Thermo Scientific™ SOLA™ hydrophobic reversed phase (HRP) plate. Analysis was carried out using a Thermo Scientific™ Syncronis™ C18 1.7 µm, 50 × 2.1 mm column to give a fast separation with a cycle time of 2 minutes while maintaining excellent peak shape.

The reproducibility of the method was measured at three concentrations for each compound and was less than 4.2% (n=6 at each level). Excellent recoveries of 94.4% (25-hydroxyvitamin D2) and 96.3% (25-hydroxyvitamin D3) were also achieved. The dynamic range was linear between 5 and 1000 ng/mL with r^2 values of 0.9994 and 0.9958 for 25-hydroxyvitamin D2 and 25-hydroxyvitamin D3, respectively.

Introduction
Vitamin D is widely monitored and used as an indicator of skeletal health in both children and adults. The active form of the hormone controls the concentration of calcium and phosphorous in the bloodstream, with deficiencies resulting in both rickets in children and osteoporosis in adults. Vitamin D occurs in two forms: vitamin D2 (ergocalciferol) is found naturally in plants and is commonly used as a dietary supplement; vitamin D3 (cholecalciferol) occurs naturally in mammals and is formed in the skin by exposure to sunlight.

Vitamin D2 and vitamin D3 are hydroxylated in the liver to form 25-hydroxyvitamin D2 (25-OHD2) (Figure 1) and 25-hydroxyvitamin D3 (25-OHD3) (Figure 2), respectively. These two forms of the compounds are then released into the circulation and transported via the vitamin D binding protein. Further hydroxylation then occurs in the kidneys to form the active hormone 1,25 dihydroxyvitamin D (1,25$(OH)_2$D). Although it is possible to monitor the active hormone, the limits of detection required to quantify this are challenging as 1,25$(OH)_2$D occurs at picogram levels within the body. It has, therefore, been readily accepted that the measurement of 25-OHD2 and 25-OHD3 is the preferred method for assessing a subjects total vitamin D status due to the higher circulating concentrations (nanogram levels) and greater serum half-life.

Suggested optimum concentration ranges within the body for 25-OH-D differs between organizations. The Endocrine Society states that a sufficient range is 30–100 ng/mL and a deficient range is 0–20 ng/mL [1]; whereas, the US Food and Nutrition board states that >20 ng/mL is sufficient and 0–11 ng/mL is classified as vitamin D deficient [2]. The dynamic range of this assay, 5 to 1000 ng/mL, allows quantification of 25-OH-D levels within a sample to show the vitamin D status of the subject.

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The extraction of 25-hydroxyvitamin D2 and 25-hydroxyvitamin D3 from human plasma is demonstrated in this application. A typical approach for sample extraction from a biological matrix is solid phase extraction (SPE). The extraction detailed here is carried out using SOLA HRP, a revolutionary SPE device. This first-in-class SPE product introduces next generation, innovative technological advancements, giving unparalleled performance characteristics compared to conventional SPE, phospholipid, and protein precipitation products.

These include:

- Higher levels of reproducibility
- Higher levels of extract cleanliness
- Reduced sample and solvent requirements
- Increased sensitivity

SOLA products provide significant advantages for the analyst when processing compounds in complex matrices, particularly in high-throughput bioanalytical and clinical research laboratories where reduced failure rate, higher analysis speed, and lower sample/solvent requirements are critical. Increased performance from SOLA products gives higher confidence in analytical results and lowers cost without compromising ease of use or requiring complex method development.

The separation of 25-hydroxyvitamin D2 and D3 is carried out using a Syncronis C18 HPLC column. One of the key goals for the chromatographer is to achieve a consistent, reproducible separation. The selection of a highly reproducible HPLC column is essential if this goal is to be attained. The Syncronis column range has been engineered to provide exceptional reproducibility due to its highly pure, high surface area silica, dense bonding, and double endcapping, all controlled and characterized through the use of rigorous testing.

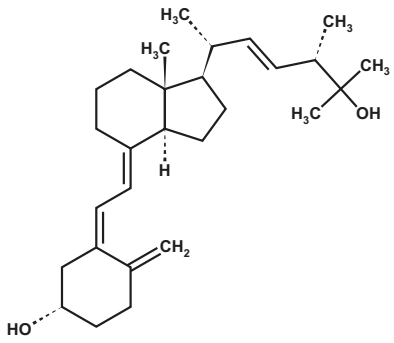

Figure 1: 25-hydroxyvitamin D2 (25-OHD2)

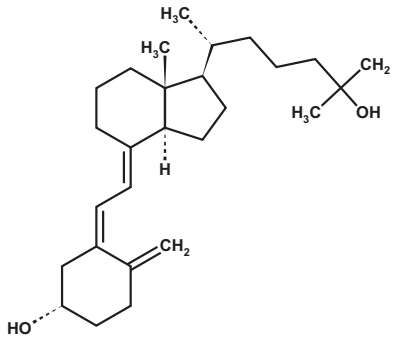

Figure 2: 25-hydroxyvitamin D3 (25-OHD3)

Experimental Details

Consumables	Part Number
Fisher Scientific™ LC/MS grade water	W/011217
Fisher Scientific LC/MS grade methanol	M/4062/17
Fisher Scientific LC/MS grade acetonitrile	A/0638/17
Fisher Scientific Analytical grade formic acid	F/1900/PB08
Thermo Scientific™ National™ Mass Spec Certified 2 mL clear vial with blue bonded PTFE/silicone cap	MSCERT4000-34W

Sample Handling Equipment	Part Number
Thermo Scientific™ 96 well plate vacuum manifold	60103-351
Thermo Scientific™ UltraVap™	60180-P900

Sample Pretreatment

The extraction of vitamin D from plasma was carried out using a SOLA HRP plate, which retained the compound by hydrophobic reversed phase interactions. As 25-OHD2 and 25-OHD3 are transported around the body by the vitamin D binding protein, it is necessary during sample pretreatment to disrupt the protein binding prior to extraction to ensure maximum compound recovery. Typically, this is carried out using 100% acetonitrile. However, as the sample is retained on the sorbent by a hydrophobic mechanism, it is important not to load in conditions that contain too much organic solvent as this will result in compound breakthrough. Therefore, acetonitrile was added to the sample prior to loading to disrupt the protein binding (ensuring that the final ratio did not exceed 1:1).

- A standard spiking solution of 25-hydroxyvitamin D2 and 25-hydroxyvitamin D3 was prepared in methanol.
- A working internal standard solution (D_6-25-hydroxyvitamin D3) was prepared in methanol.
- To 200 µL of blank human plasma, 180 µL of acetonitrile was added.
- For standards and quality control (QC) samples, 10 µL of standard spiking solution was added.
- For standards and QCs, 10 µL of working internal standard solution was added.
- For blanks, 10 µL of methanol only was added.
- All samples were vortexed for 30 seconds and then centrifuged for 5 minutes at 5000 rpm.

Sample Preparation		Part Number
During method development, it was observed that up to 50% methanol could be applied to the SPE plate without causing the compounds to be eluted. Therefore, the wash solution used to remove matrix interferences was 40% methanol. To elute the compounds of interest, 100% methanol was applied to ensure maximum recovery.		
Compound(s):	25-hydroxyvitamin D2, 25-hydroxyvitamin D3, D6-25-hydroxyvitamin D3 (IS)	
Matrix:	Human plasma	
Plate type:	Thermo Scientific SOLA HRP 10 mg/2 mL	60309-001
Conditioning:	0.5 mL methanol, then 0.5 mL water	
Application:	200 µL of sample supernatant	
Wash:	200 µL water / methanol (60:40 v/v)	
Elution:	2 × 200 µL methanol	
Dry down:	Under nitrogen without heat	
Reconstitute:	In 100 µL water/ acetonitrile (30:70 v/v), mix well	

Separation Conditions		Part Number
A Syncronis C18 HPLC column was used for the analysis. The Syncronis product range is based on a high surface area silica, which results in extra retention of analytes in comparison to chromatography columns with a lower surface area. This was significant within the vitamin D application, as additional retention was required to shift the peaks of interest away from isobaric interferences to increase sensitivity.		
Recommended instrumentation:	Thermo Scientific™ Dionex™ UltiMate™ 3000 HPLC system	
Column:	Syncronis C18, 1.7 µm, 50 x 2.1 mm	97102-052130
Mobile phase A:	Water + 0.1% formic acid	
Mobile phase B:	Acetonitrile + 0.1% formic acid	
Gradient:	70–100% B in 2 minutes	
Flow rate (mL/min):	0.8	
Column temperature (°C):	40	
Injection (µL):	50	
Injection wash solvent 1:	Water / acetonitrile (80:20 v/v)	
Injection wash solvent 2:	IPA / acetonitrile / acetone (45:45:10 v/v/v)	

MS Conditions	
Instrumentation:	Thermo Scientific™ TSQ Vantage™ triple stage quadrupole mass spectrometer
Ionization conditions:	APCI
Polarity:	Positive
Discharge current:	4.0
Vaporizer temp (°C):	400
Sheath gas pressure (Arb):	25
Aux gas pressure (Arb):	10
Capillary temp (°C):	275
Collision pressure (mTorr):	1.5
Scan time (s):	0.02
Q1 (FWHM):	0.7
Q3 (FWHM):	0.7
Transition details:	Refer to Table 1

Compound	25-hydroxyvitamin D2		25-hydroxyvitamin D3		D_6-25-hydroxyvitamin D3 (IS)
Parent (*m/z*)	395.4		383.3		389.3
Products (*m/z*)	209.2	269.2	211.2	365.4	371.4
Collision energy	27	19	23	11	12
S-lens	102	102	91	91	77

Table 1: Compound transition details

Data Processing	
Software:	Thermo Scientific™ LCQUAN™ version 2.6

Results

Extracted 25-hydroxyvitamin D2 standards from human plasma gave a linear calibration curve over the dynamic range of 5 to 1000 ng/mL with an r^2 coefficient of 0.9994 (Figure 3 and Table 2). The chromatography of the LOQ at 5 ng/mL is shown in Figure 4. QC samples were run in replicates of six at concentrations of 15, 250, and 600 ng/mL. Precision at each of the QC levels was less than 4% RSD (Table 3). Overspikes were analyzed at concentrations of 15, 250, and 600 ng/mL and used to calculate recovery. The overall percentage recovery level of 25-hydroxyvitamin D2 of 94.4% was calculated using the average recovery at the three QC levels (Table 4).

Extracted 25-hydroxyvitamin D3 standards from human plasma gave a linear calibration curve over the dynamic range of 5 to 1000 ng/mL with an r^2 coefficient of 0.9958 (Figure 5 and Table 2). The chromatography of the limit of quantitation of 5 ng/mL is shown in Figure 6. QC samples were run in replicates of six at concentrations of 15, 250, and 600 ng/mL. The precision at each of the QC levels was less than 4.2% RSD (Table 5). Overspikes were analyzed at concentrations of 15, 250, and 600 ng/mL and used to calculate recovery. The overall percentage recovery level of 25-hydroxyvitamin D3 of 96.3% was calculated using the average recovery of the three QC levels (Table 6).

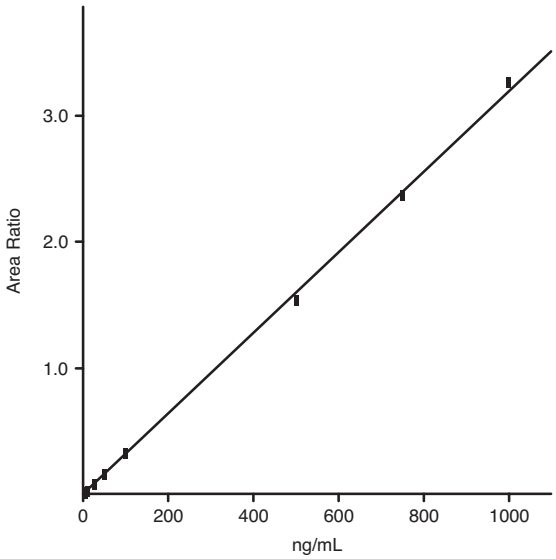

Figure 3: 25-hydroxyvitamin D2 linearity over the dynamic range 5–1000 ng/mL

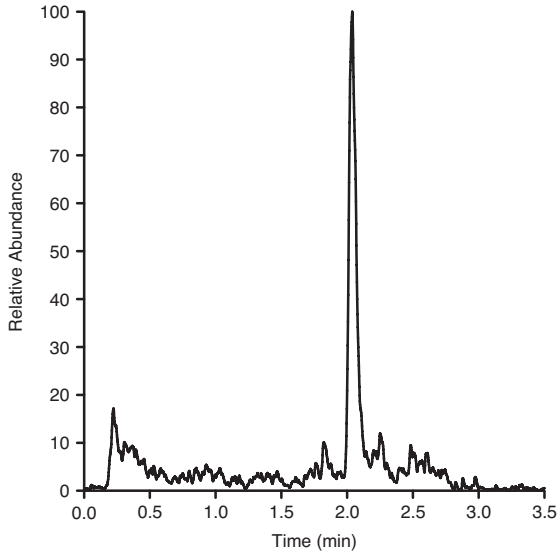

Figure 4: Representative chromatogram of 25-hydroxyvitamin D2 SRM, extracted from human plasma at 5 ng/mL

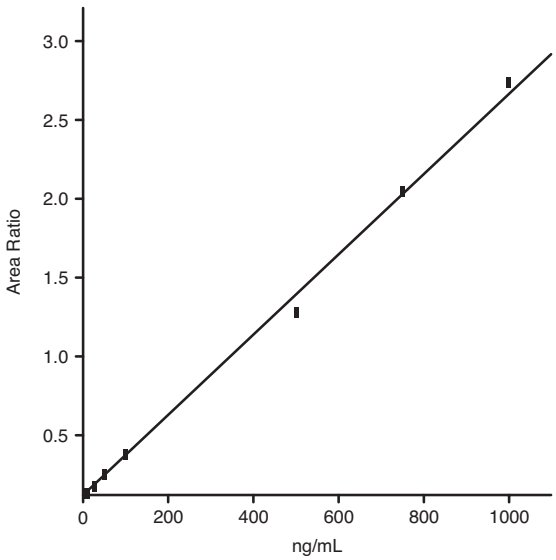

Figure 5: 25-hydroxyvitamin D3 linearity over the dynamic range 5–1000 ng/mL

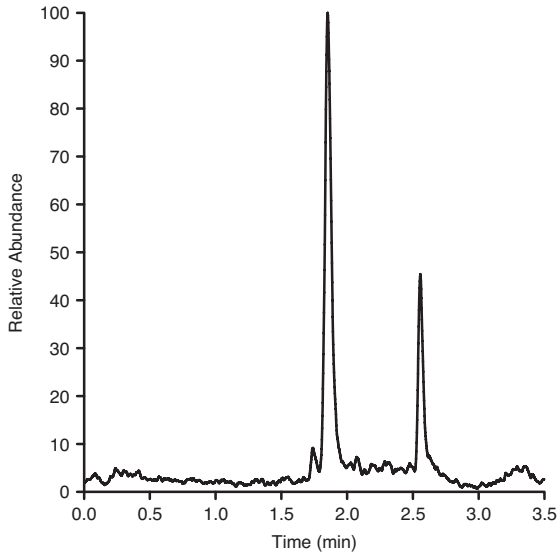

Figure 6: Representative chromatogram of 25-hydroxyvitamin D3 SRM, extracted from human plasma at 5 ng/mL

Standard	Analyte	Specified Concentration (ng/mL)	Calculated Concentration (ng/mL)	%Diff
S1	25-hydroxyvitamin D2	5	5.15	3
S1	25-hydroxyvitamin D2	5	4.88	-2
S2	25-hydroxyvitamin D2	10	9.81	-2
S3	25-hydroxyvitamin D2	25	25.2	1
S4	25-hydroxyvitamin D2	50	50.7	1
S5	25-hydroxyvitamin D2	100	101	1
S6	25-hydroxyvitamin D2	500	482	-4
S7	25-hydroxyvitamin D2	750	743	-1
S8	25-hydroxyvitamin D2	1000	1023	2
S1	25-hydroxyvitamin D3	5	4.96	-1
S1	25-hydroxyvitamin D3	5	5.56	11
S2	25-hydroxyvitamin D3	25	22.8	-9
S3	25-hydroxyvitamin D3	50	53.1	6
S4	25-hydroxyvitamin D3	100	98.6	-1
S5	25-hydroxyvitamin D3	500	441	-12
S6	25-hydroxyvitamin D3	750	734	-2
S7	25-hydroxyvitamin D3	1000	996	0
S7	25-hydroxyvitamin D3	1000	1078	8

Table 2: Accuracy data for extracted standards over the linear range 5–1000 ng/mL

Standard	Concentration (ng/mL)	Average Response (n=6)	Precision (%CV)
QCL	15	25,105	3.7
QCM	250	402,130	2.6
QCH	600	1,131,285	3.4

Table 3: Average precision data for six replicate QCs for 25-hydroxyvitamin D2

Standard	Response	% Recovery at each level	% Recovery
Average QCL area response	25,105	93.8	94.4
Average overspike area response	26,774		
Average QCM area response	402,130	91.5	
Average overspike area response	439,544		
Average QCH area response	1,131,285	97.8	
Average overspike area response	1,156,632		

Table 4: Recovery data for 25-hydroxyvitamin D2

Standard	Concentration (ng/mL)	Average Response (n=6)	Precision (%CV)
QCL	15	77,549	3.4
QCM	250	387,542	2.7
QCH	600	938,040	4.2

Table 5: Average precision data for six replicate QCs for 25-hydroxyvitamin D3

Standard	Response	% Recovery at each level	% Recovery
Average QCL area response	77,549	90.8	96.3
Average overspike area response	85,371		
Average QCM area response	387,542	96.7	
Average overspike area response	400,888		
Average QCH area response	938,040	101.3	
Average overspike area response	925,778		

Table 6: Recovery data for 25-hydroxyvitamin D3

Conclusion

- The use of SOLA HRP SPE and a Syncronis C18 UHPLC column allows for simple extraction and rapid quantification of 25-hydroxyvitamin D2 and 25-hydroxyvitamin D3 from human plasma.
- A limit of quantitation of 5 ng/mL for both compounds in plasma was achieved.
- Extraction recovery was >94% and >96% for 25-hydroxyvitamin D2 and 25-hydroxyvitamin D3, respectively.
- The method showed good precision with %RSD (n=6) <4.2% for both compounds.

References

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