The Analysis of Vitamin D Metabolites in Serum/Plasma by LC-MS/MS

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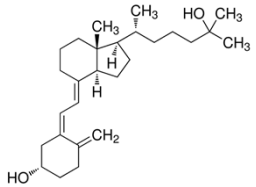
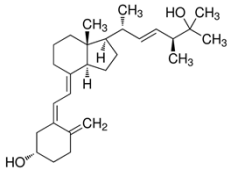
Abstract & Introduction

Vitamin D deficiency has been linked to an increased risk for many chronic diseases including diabetes, heart disease, autoimmune diseases, and some cancers. Vitamin D exists in two forms, vitamin D_2 and vitamin D_3. Each undergoes metabolism to form 25-hydroxyvitamin D_2 and 25-hydroxyvitamin D_3. For accurate determination of vitamin D levels in the blood, it is important to distinguish between these metabolites and to separate them from major matrix interferences. In this method, the Raptor™ ARC-18 column combines the speed of superficially porous particles (SPP) with the resolution of highly selective USLC® technology to produce a simple and rugged method for the determination of vitamin D metabolites in serum and plasma.



25-hydroxyvitamin D3



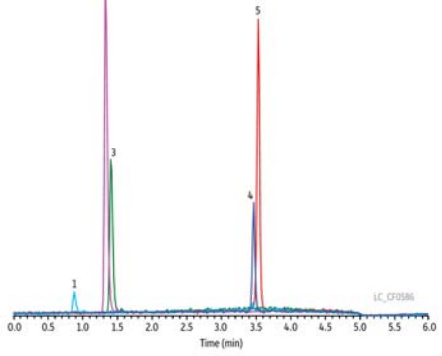
25-hydroxyvitamin D2

The suitability of ARC-18 column for the analysis of vitamin D metabolites was first demonstrated for the analysis of neat standard solution containing fat-soluble vitamin D and metabolites. The ARC-18 was then used to analyze the 25-hydroxyvitamin D metabolites of fortified serum (Beagle) and plasma (Rat). For the purpose of this presentation, only data from the plasma extraction is shown. The quantitation was performed with calibrated standards prepared in 4% human albumin PBS solution. Three levels of fortified metabolite concentration were measured with acceptable accuracy and precision.

Analysis of Vitamin D and Metabolites with Raptor™ ARC-18

Figure 1: The Raptor ™ ARC-18 Makes Quick Work of Fat-Soluble Vitamin D and Metabolites by LC-MS/MS

Peaks	t, (min)	Conc. (ng/mL)	Q1	Q3
1. 1,25-Dihydroxyvitamin D3	0.88	200	399.4	381.5
2. 25-Hydroxyvitamin D3	1.33	200	401.5	383.5
3. 25-Hydroxyvitamin D2	1.41	200	413.5	395.5
4. Vitamin D2	3.47	200	397.5	379.6
5. Vitamin D3	3.53	200	385.5	367.5



Column: Raptor™ ARC-18 (cat.# 9314A12); Dimensions: 100 mm x 2.1 mm ID; Particle Size: 2.7 µm; Temp.: 40 °C; **Sample:** Diluent: Methanol; Conc.: 200 ng/mL; Inj. Vol.: 5 µL; **Mobile Phase:** A: 0.1% Formic acid + 5 mM ammonium formate in water B: 0.1% Formic acid + 5 mM ammonium formate in methanol; **Gradient (%B):** 0.00 min (90%), 4.00 min (100%), 4.01 min (90%), 6.00 (90%); **Flow:** 0.5 mL/min; **Detector:** ABSCIEX API 4000™; Ion Source: TurbolonSpray®; Ion Mode: ESI+; **Instrument:** Shimadzu UFLC XR

Separating fat-soluble vitamins by LC can be time consuming. The Raptor™ ARC-18 column, however, can analyze these difficult compounds using reversed-phased chromatography (RPC) in less time than traditional columns to increase productivity. Plus, in the bioanalytical arena, the ARC-18 can quantitate the metabolites of vitamin D using the same column and mobile phases.

Sample Preparation

The metabolites were fortified into Rat plasma and extracted with a liquid-liquid extraction method. Plasma (150µL) was mixed with 0.2M $ZnSO_4$ (150µL) in a 2-mL glass HPLC vial. Added 300µL of methanol containing 25ng/mL of d6-25(OH)D3 (internal standard) and vortex mixed (10secs). Added 750µL of hexane, mixed for 30secs, and then centrifuged for 10mins at 4300rpm. The hexane layer (~650µL) was removed and placed into the micro-vial and evaporated to dryness under nitrogen at 55°C. The dried extract was reconstituted with 75µL of 15:85 water:methanol solution and injected (5 µL) for analysis.

Calibration Standards and Fortified QC Samples

The human albumin was dissolved in PBS solution at the final concentration of 4%. This solution was used to prepare the 25(OH)D2 and 25(OH)D3 calibration standard ranged from 1 to 150 ng/mL. Three levels of QC samples were prepared by fortification of 25(OH)D2 and 25(OH)D3 into and Rat plasma at 5, 25, and 100ng/mL.

Methods

Table 1: Analytical Conditions for AB SCIEX TripleQuad 4500 with Shimadzu Nexera X2

Analytical Column	Raptor™ ARC-18, 100 mm x 2.1 mm, 2.7 µm	
Mobile Phase A	0.1% formic acid in water	
Mobile Phase B	0.1% formic acid in methanol	
Gradient	**Time (min)**	**%B**
	0.0	85
	3.0	96
	3.01	96
	5.0	96
Flow Rate	0.5 mL/min	
Run Time	5.0 min	
Column Temp.	40°C	
Ion Mode	Positive ESI (5000)	
CAD	5	
CUR	20	
GS1	30	
GS2	55	
Source Temp.	250°C	

Table 2: Analyte Transitions

Analyte	Precursor Ion	Product Ion Quantifier	Product Ion Qualifier
25-hydroxyvitamin D2	413.3	395.5	355.4
25-hydroxyvitamin D3	401.3	383.5	365.4
d6-25-hydroxyvitamin D3	407.3	389.5	NA

Chromatograms

Figure 2: Chromatograms of Standard Solutions

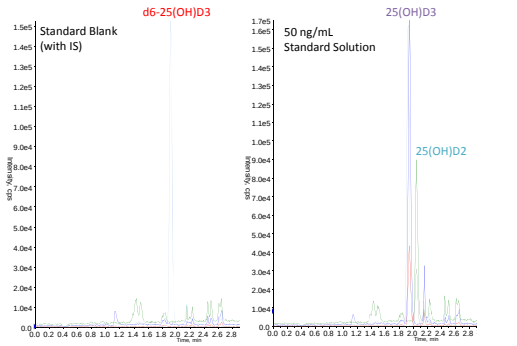
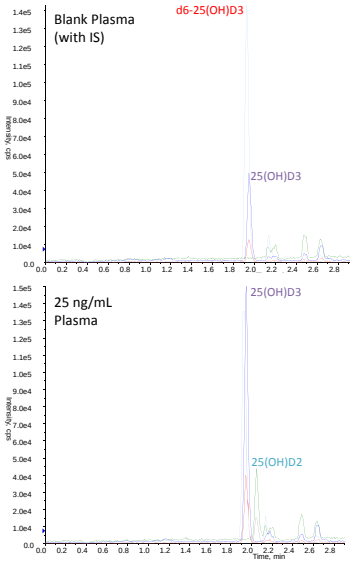


Figure 3: Chromatograms of Fortified Rat Plasma



Both Beagle serum and Rat plasma contain 25(OH)D3 as shown in *Figure 3* for the extracted blank plasma. Therefore, the quantity of 25(OH)D3 was determined by subtraction of the calculated 25(OH)D3 concentration in the fortified sample by that in the blank plasma.

Results

The calibration standards were prepared in 4% human albumin in PBS solution and subjected to the same extraction procedure for the fortified plasma samples (see *Figure 2* for chromatograms). A good linearity was obtained for both 25(OH)D2 and 25(OH)D3 from 1 to 150ng/mL (with 1/x weighting). The r was ~0.999 and the %deviation was ≤10%. (*Figure 4*). *Table 3* shows the quantitation result of 3 levels of fortified plasma samples.

Figure 4: Linearities

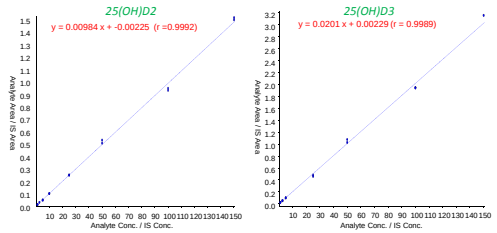


Table 3: Quantitation of Vitamin D Metabolites in Plasma

	Low Fortified Conc. (5 ng/mL)			Medium Fortified Conc. (25 ng/mL)			High Fortified Conc. (100 ng/mL)		
Analyte	Avg. Conc. (ng/mL)	Avg. Accuracy	%RSD	Avg. Conc. (ng/mL)	Avg. Accuracy	%RSD	Avg. Conc. (ng/mL)	Avg. Accuracy	%RSD
25(OH)D2	5.4	107.3	10.7	25.3	101.1	3.9	101.5	101.5	1.6
25(OH)D3	4.5	92.6	8.5	25.6	102.4	0.3	107.1	107.1	1.4

Conclusions

Raptor™ LC columns offer the speed of superficially porous particles with the resolution of the highly selective USLC® stationary phases. This presentation demonstrated that the Raptor™ ARC-18 column is suitable for the measurement of vitamin D metabolites in serum and plasma with fast analysis speed and acceptable accuracy and precision.