APPLICATION NOTE

Quantitation of 25-Hydroxyvitamin D2 and 25-Hydroxyvitamin D3 in Plasma for Clinical Research Using the Prelude SPLC and TSQ Endura Mass Spectrometer

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Key Words
25-hydroxyvitamin D2, 25-hydroxyvitamin D3, Prelude SPLC, TSQ Endura MS

Application Benefits

- Easy and economical online sample cleanup
- Throughput of 24 injections per hour on two channels
- Linearity range: 4–200 ng/mL for individual analyte
- Good method precision (<7% RSD) and accuracy (93%–106% recovery)

Goal

To evaluate the performance of the Thermo Scientific™ Prelude SPLC™ system and Thermo Scientific™ TSQ Endura™ mass spectrometer as a quantitative platform for LC-MS analysis of 25-hydroxyvitamin D2 and 25-hydroxyvitamin D3 in human plasma for clinical research.

Introduction

LC-MS methods have been widely used to analyze vitamin D2 and vitamin D3 25-hydroxy metabolites (25OH D2 and 25OH D3) in human plasma. Clinical research laboratories are always seeking fast and cost-effective methods to improve analytical efficiency. Here we evaluated a high-efficiency, simple sample preparation method implemented on a Prelude SPLC system that combines online sample cleanup powered by Thermo Scientific™ TurboFlow™ technology with chromatographic separation and a TSQ Endura triple quadrupole mass spectrometer.

The Prelude SPLC system features two independent channels of sample preparation and liquid chromatography. Thus, the chromatographic methods on the Prelude SPLC system can be executed in parallel, either with a different method on each channel or the same method on both channels. The two-channel operation on the Prelude SPLC system is automatically optimized into one mass spectrometer for serial detection, which improves mass spectrometer utilization time, increases throughput, and reduces analysis cost.

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Methods

Sample Preparation

Plasma samples were processed by protein precipitation. Briefly, 200 µL of methanol containing internal standard (d6-25OH D3) was added to 100 µL of sample (calibrators, controls, or unknowns). The resulting mixture was vortexed and centrifuged, and 100 µL was injected for LC-MS/MS analysis.

Calibration Standards

Calibration standards at concentrations of 4, 10, 25, 50, 100, and 200 ng/mL were prepared in ethanol because analyte-free plasma was not available. Data collected for National Institute of Standards and Technology (NIST) controls and spiked plasma recovery experiments were used to demonstrate that calibrators prepared in solvent are a valid surrogate for plasma matrix.

Quality Control (QC) Samples

QC samples (Table 1) were prepared by spiking 5, 20, and 100 ng/mL analytes into previously analyzed pooled donor plasma.

Table 1. Concentrations of 25-hydroxy vitamin D2 and 25-hydroxy vitamin D3 in in-house-prepared QC samples.

Analyte	QC0	QC1	QC2	QC3
	Concentration (ng/mL)			
25OH D2	0.0	5.0	20.0	100.0
25OH D3	25.2	30.2	45.2	125.2

Liquid Chromatography

The processed sample was directly injected onto a Thermo Scientific™ Cyclone-P™ column (0.5 X 50 mm, P/N CH-953289) for online sample cleanup. This step was followed by chromatographic separation on a Thermo Scientific™ Accucore™ aQ column (2.1 X 100 mm, 2.6 µm, P/N 17326-102130). The column temperature was set at 25 °C. The total run time was five minutes (Figure 1).

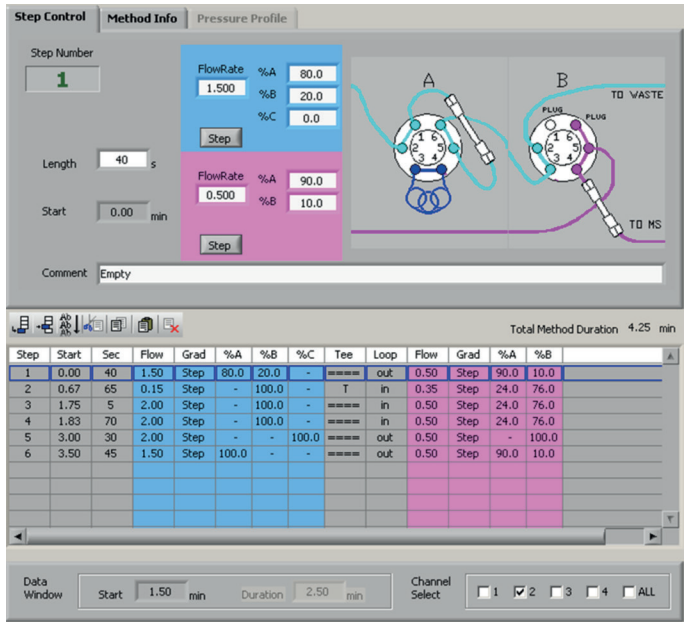


Figure 1. Prelude SPLC method.

Mass Spectrometry

Compounds were detected on a TSQ Endura triple quadrupole mass spectrometer equipped with atmospheric pressure chemical ionization (APCI) probe. Data were acquired in selected-reaction monitoring (SRM) mode.

Data Analysis

Data were acquired and processed using Thermo Scientific™ TraceFinder™ software.

Method Performance Evaluation

The limit of quantitation (LOQ) and linearity range were evaluated by collecting calibration curve data. Method precision was evaluated by running five replicates of QCs on five different days. Recovery was evaluated by spiking 20 ng/mL of each analyte to ten different plasma samples analyzed in quintuplicate. Matrix effects were evaluated by spiking 20 ng/mL of each analyte into ten different donor samples and water. Absolute matrix effect was computed by dividing the analyte peak area of donor samples by the peak area of water, expressed as percent. Relative matrix effect was computed by dividing the analyte peak area ratio against internal standard of donor samples by the peak area ratio of water, expressed as percent. Accuracy was evaluated by analyzing the standards from NIST.

Results

LOQs were defined as the lowest concentrations that had back-calculated values within 20%. Using these criteria, the limits of quantitation for 25OH D2 and 25OH D3 were 4 ng/mL in human plasma.

Figure 2 shows representative chromatograms for internal standards and analytes at their respective LOQs. Calibration ranges were determined to be 4–200 ng/mL, where 200 ng/mL was the highest concentration evaluated. Figure 3 shows calibration curves for all analytes and chromatograms for the lowest calibration standard.

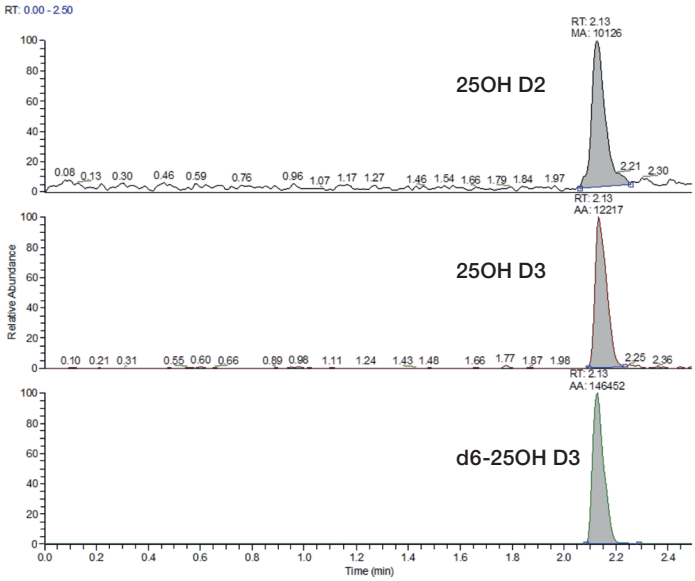
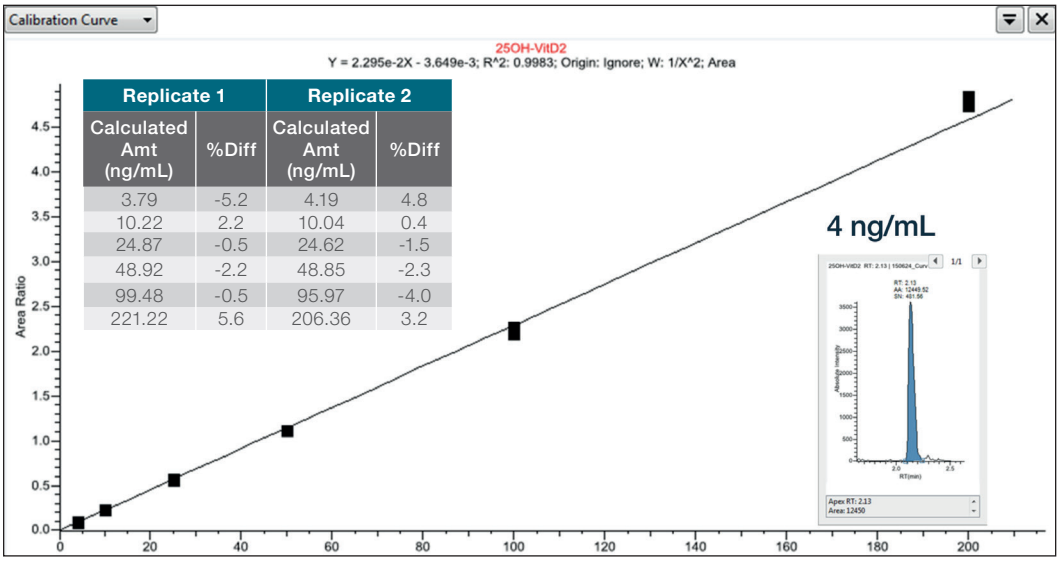
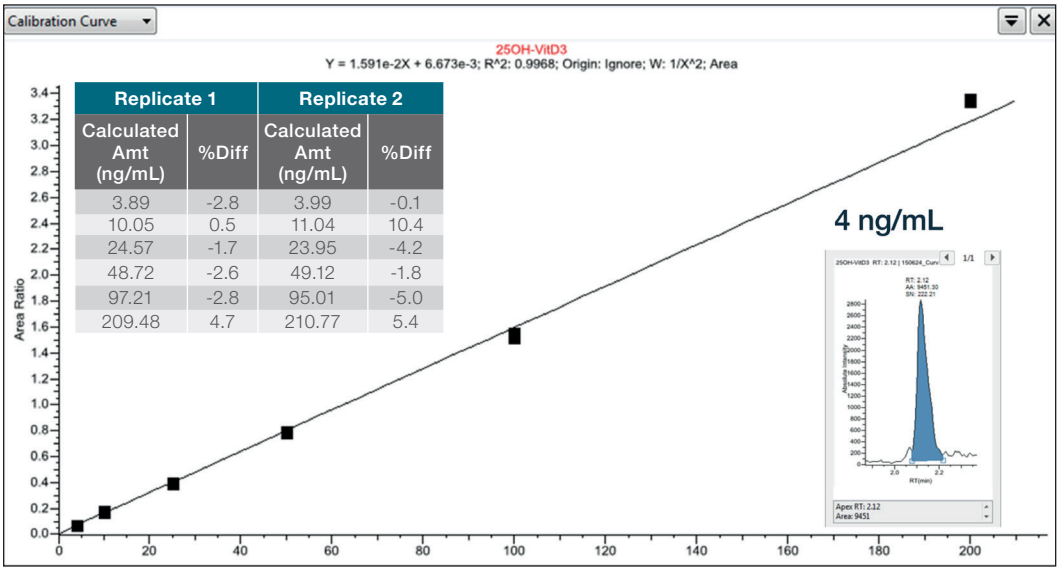


Figure 2. Representative chromatograms of the lowest calibration standard (4 ng/mL).



Replicate 1		Replicate 2	
Calculated Amt (ng/mL)	%Diff	Calculated Amt (ng/mL)	%Diff
3.79	-5.2	4.19	4.8
10.22	2.2	10.04	0.4
24.87	-0.5	24.62	-1.5
48.92	-2.2	48.85	-2.3
99.48	-0.5	95.97	-4.0
221.22	5.6	206.36	3.2

Figure 3a. Calibration curve for 25OH D2.



Replicate 1		Replicate 2	
Calculated Amt (ng/mL)	%Diff	Calculated Amt (ng/mL)	%Diff
3.89	-2.8	3.99	-0.1
10.05	0.5	11.04	10.4
24.57	-1.7	23.95	-4.2
48.72	-2.6	49.12	-1.8
97.21	-2.8	95.01	-5.0
209.48	4.7	210.77	5.4

Figure 3b. Calibration curve for 25OH D3.

Inter-assay precision was better than 5.1% RSD and 6.6% RSD for 25OH D2 and 25OH D3, respectively (Table 2).

Table 2. Inter-assay precision for QC samples.

Analyte	QC0	QC1	QC2	QC3
	%RSD			
25OH D2	--	5.1	2.3	3.0
25OH D3	6.6	3.1	2.4	1.8

Recovery rate ranged from 107% to 132% for 25OH D2 and from 102% to 118% for 25OH D3 (Table 3).

Table 3. Recovery rate.

Analyte	Plasma 1	Plasma 2	Plasma 3	Plasma 4	Plasma 5	Plasma 6	Plasma 7	Plasma 8	Plasma 9	Plasma 10
	% Recovery									
25OH D2	114	108	107	119	132	124	127	119	119	120
25OH D3	118	115	104	112	114	110	113	100	112	102

The absolute matrix effect ranged from 62.6% to 79.0% for 25OH D2 and from 61.4% to 79.0% for 25OH D3. The ion suppression for 25OH D3 was greatly corrected by the addition of an internal standard: the relative matrix effect ranged from 98.3% to 116%. Matrix effects for 25OH D2 were partially corrected by using internal standard: the relative matrix effect ranged from 75.9% to 89.4%. Based on these data, the use of deuterated 25OH D2 as internal standard for analysis of 25OH D2 is recommended. Matrix effect data is summarized in (Table 4).

Method accuracy and matrix equivalence, as determined by analysis of NIST Standard Reference Material ranged from 93.3% to 107% for 25OH D3. The accuracy of the single standard concentration was 97.0% for 25OH D2 (Table 5).

Table 4. Matrix effect.

Analyte	Plasma 1	Plasma 2	Plasma 3	Plasma 4	Plasma 5	Plasma 6	Plasma 7	Plasma 8	Plasma 9	Plasma 10
	Absolute Matrix Effect (%)									
25OH D2	79.0	66.6	62.6	68.7	66.5	66.9	68.6	66.6	69.1	65.7
25OH D3	79.0	64.3	64.4	65.3	61.5	63.4	67.0	62.6	67.8	61.4

Analyte	Plasma 1	Plasma 2	Plasma 3	Plasma 4	Plasma 5	Plasma 6	Plasma 7	Plasma 8	Plasma 9	Plasma 10
	Relative Matrix Effect (%)									
25OH D2	89.4	83.1	78.8	77.7	75.9	84.0	83.1	84.2	81.1	80.5
25OH D3	116	104	101	99.3	98.3	107	106	107	103	103

Table 5. Recovery of NIST standards calculated against a calibration curve prepared in ethanol, showing matrix equivalence and method accuracy.

	25OH D3			25OH D2		
	Expected (ng/mL)	Obtained (ng/mL)	% Recovery	Expected (ng/mL)	Obtained (ng/mL)	% Recovery
Level 1	30.6	32.9	107	-	-	-
Level 2	19.4	20.3	105	-	-	-
Level 3	21.0	21.5	103	13.3	12.9	97.0
Level 4	55.8	52.1	93.3	-	-	-

Conclusion

We demonstrated a simple, high-efficiency method for analysis of 25-hydroxyvitamin D2 and 25-hydroxyvitamin D3 in human plasma using the Prelude SPLC system and TSQ Endura mass spectrometer for clinical research applications. The method evaluation results met clinical research lab requirements. The Prelude SPLC system provides automated online sample cleanup in addition to offline preparation and two-channel operation, thus improving method robustness and instrument uptime and increasing sample throughput (24 samples/hour on two channels).

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