Quantitation of 25-Hydroxyvitamin D3 and 25-Hydroxyvitamin D2 in Plasma for Clinical Research Using an Affordable High-Resolution Mass Spectrometer

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Application Note 634

Key Words
Q Exactive Focus, vitamin D, clinical research

Goal
To evaluate the performance of the Thermo Scientific™ Q Exactive™ Focus high-resolution Orbitrap mass spectrometer as a quantitative platform for HPLC-MS analysis of 25-hydroxyvitamin D metabolites in human plasma for clinical research.

Application Benefits
- Simple sample preparation
- Short analysis time
- High selectivity
- Limited matrix effects
- Accurate chromatographic peak integration due to very low background
- Method performance meets clinical research requirements
- Method accuracy proved with NIST calibrators

Introduction
Analysis of vitamin D2 and vitamin D3 25-hydroxy metabolites (25OHD2 and 25OHD3) in human plasma is one of the highest volume clinical research applications that utilize LC-MS methods. Clinical research laboratories are seeking fast and cost-efficient methods to improve analytical efficiency. Here we evaluated a high-efficiency, simple sample preparation method implemented on a Q Exactive Focus hybrid quadrupole-Orbitrap mass spectrometer for improved selectivity when compared to a triple quadrupole mass spectrometer

Methods
Sample Preparation
Samples were processed by protein precipitation. Briefly, 300 µL of acetonitrile/methanol 9:1 (v:v) containing internal standard (D_6–25OHD3) was added to 100 µL of plasma (calibrator, control, or unknown). The mixture was vortexed and centrifuged, and supernatant was injected onto the analytical column.

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

QC Samples
QC samples (Table 1) were prepared by spiking previously analyzed pooled donor plasma.

Liquid Chromatography
Gradient elution was performed using a Thermo Scientific™ Dionex™ UltiMate™ 3000RS with OAS autosampler. Mobile phases consisted of 0.1% formic acid in water and methanol (Fisher Chemical™ Optima™ grade solvent) for solvents A and B, respectively. The column used was a Thermo Scientific™ Hypersil GOLD™ aQ, 5 µm, 50 x 2.1 mm column (P/N 25305-052130). The total run time was two minutes.

Mass Spectrometry
Compounds were detected on a Q Exactive Focus Orbitrap benchtop mass spectrometer equipped with a Thermo Scientific™ Ion Max™ source and an atmospheric pressure chemical ionization (APCI) probe. Data were acquired in parallel-reaction monitoring (PRM) mode. In this mode, a single precursor ion is selected in the quadrupole with an isolation width of 2.0 *m/z* and fragmented in the higher-energy collisional dissociation (HCD) cell using optimized compound specific collision energy. The resulting MS/MS product ion spectrum was detected in the Thermo Scientific™ Orbitrap™ detector at a resolution of 17,500 (FWMH at 200 *m/z*).

Table 1. Concentratons of vitamin D2(25OHD) and vitamin D3(25OHD3) in QC samples.

Analyte	QC1	QC2	QC3
	Concentraton (ng/mL)		
Vitamin D2 (25OHD2)	10.0	20.0	30.0
Vitamin D3 (25OHD3)	21.5	39.6	60.2

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Method Evaluation

The limit of quantitation (LOQ) and linearity range were evaluated by collecting calibration curve data in quintuplicate. Method precision and accuracy were evaluated by running a calibration curve and quintuplicate replicates of quality controls on three different days. Matrix effects were evaluated by spiking 15 ng/mL of each analyte in triplicate into previously analyzed plasma from six different donors and calculating average %Recovery.

Data Analysis

Data were acquired and processed using Thermo Scientific™ TraceFinder™ software version 3.2. For each analyte, a specific fragment from the MS/MS spectrum was selected as the quantifying ion. The resulting chromatograms were extracted and reconstructed with a mass accuracy of 5 ppm for quantification.

Results

Limits of quantitation (LOQs) were defined as the lowest concentrations that had back-calculated values within 20% and %RSD for five replicates within 20%. Using these criteria, limits of quantitation for 25OHD3 and 25OHD2 were determined to be 4 ng/mL. Figure 1 shows a combined stick mode chromatogram for the internal standard and analytes at their respective LOQs illustrating over 20 scans collected across the peak.

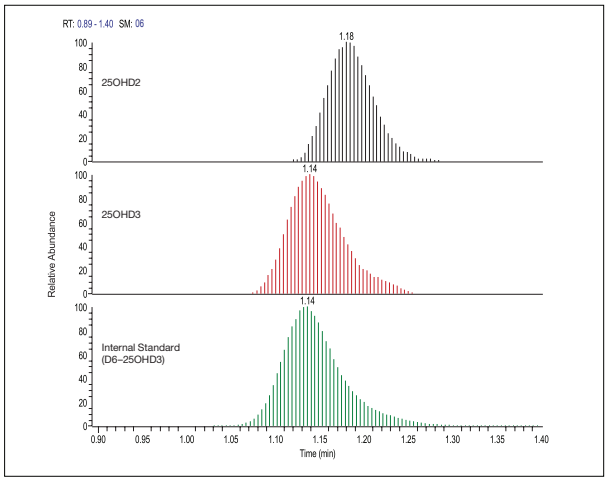

Figure 1. Chromatogram of the lowest calibraton standard (4 ng/mL) reconstructed with mass accuracy of 5 ppm.

Calibration ranges were determined to be 4–100 ng/mL, where 100 ng/mL was the highest evaluated concentration. Figure 2a and Figure 2b show representative calibration curves for both analytes and chromatograms for the lowest calibration standard.

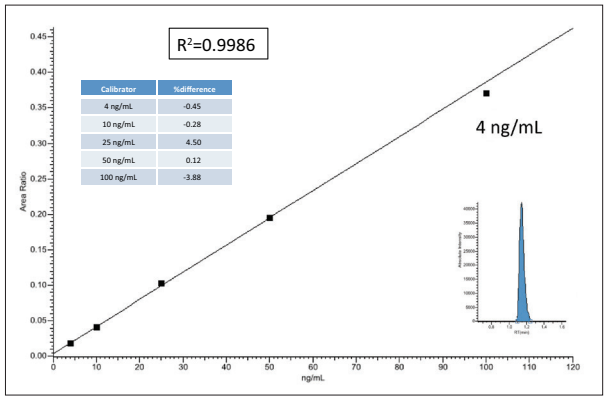

Figure 2a. Representative calibration curve for 25-hydroxyvitamin D3

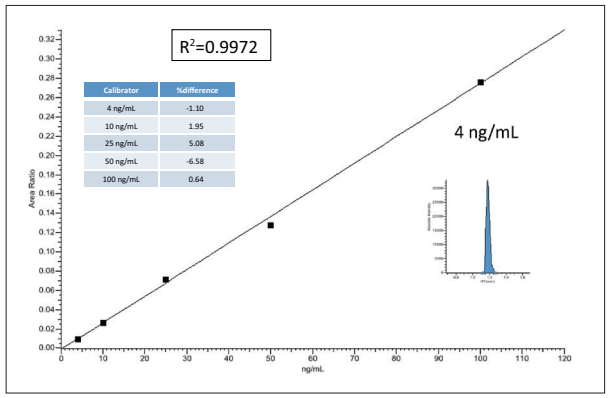

Figure 2b. Representative calibration curve for 25-hydroxyvitamin D2.

Intra-assay precision was better than 7.64% RSD and 13.0% RSD for 25OHD3 and 25OHD2, respectively (Table 2).

Table 2. Intra-assay precision data.

Analyte	QC1	QC2	QC3
	%RSD		
Vitamin D2 (25OHD2)	6.3–12.7	5.3–13.0	2.8–5.3
Vitamin D3 (25OHD3)	3.2–7.6	2.6–5.9	3.7–4.7

Inter-assay precision was better than 6.1% RSD and 10.5 %RSD for 25OHD3 and 25OHD2, respectively (Table 3).

Table 3. Inter-assay precision data.

Analyte	QC1	QC2	QC3
	%RSD		
Vitamin D2 (25OHD2)	10.5	9.0	4.5
Vitamin D3 (25OHD3)	5.6	3.9	6.1

Method accuracy determined by analysis of NIST calibrators for 25OHD3 ranged from 88.3% to 100%. For 25OHD2, the accuracy of the single calibration concentration was 85.8% (Table 4).

Table 4. NIST calibrators %Recovery. Deuterated analog of 25OHD3 was used as internal standard for both analytes.

Calibrator	Vitamin D3 (25OHD3)			Vitamin D2 (25OHD3)		
	Expected (ng/mL)	Obtained (ng/mL)	% Recovery	Expected (ng/mL)	Obtained (ng/mL)	% Recovery
Level 1	30.6	29.2	95.3	—	—	—
Level 2	19.4	17.7	91.5	—	—	—
Level 3	21.0	21.1	100	13.3	11.4	85.8
Level 4	55.8	49.3	88.3	—	—	—

Limited matrix effects were observed. %Recovery in six donor samples ranged from 104% to 112% and from 79.9% to 91.2% for 25OHD3 and 25OHD2, respectively. Figure 3 shows representative chromatograms for selected spiked donor samples.

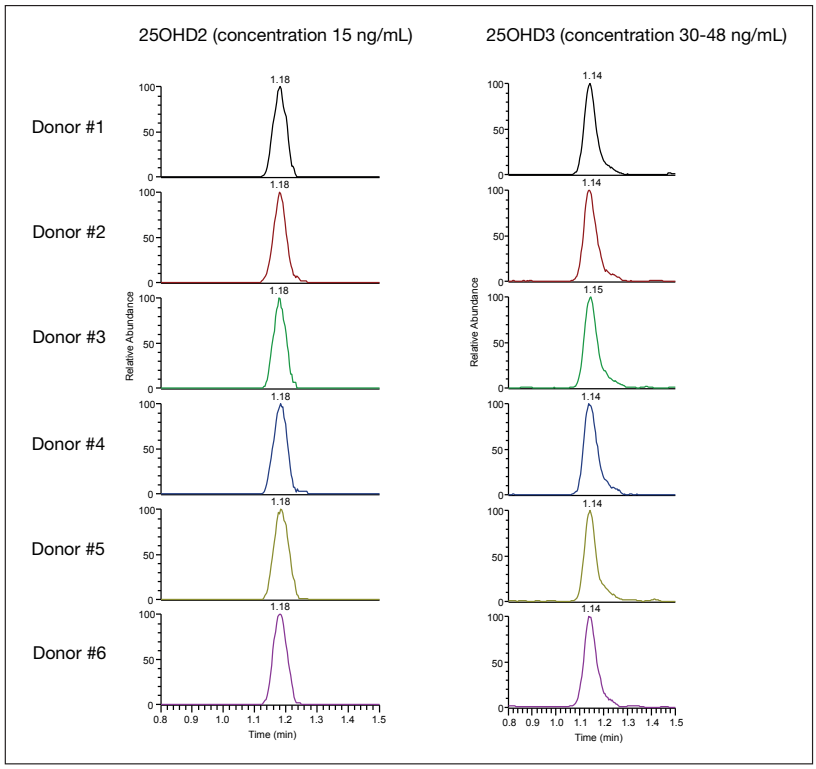

Figure 3. Chromatograms of 25-hydroxyvitamin D2 and 25-hydroxyvitamin D3 in donor plasma samples showing no interfering peaks.

Method efficiency can be improved four times with the four-channel Thermo Scientific™ Transcend™ II LC system (Figure 4).

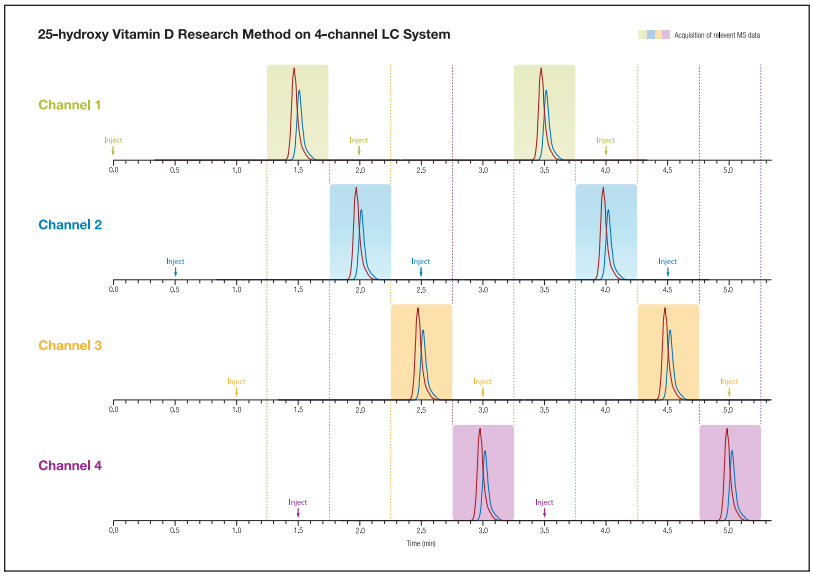

Figure 4. Execution of the method on a four-channel LC system.

Conclusion

We demonstrated a simple, high-efficiency method for the analysis of 25-hydroxyvitamin D3 and 25-hydroxyvitamin D2 in human plasma implemented on a Q Exactive Focus high-resolution mass spectrometer for clinical research applications. The method evaluation results met clinical research lab requirements.

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