Application Note 636

Rapid Analysis of 25-Hydroxyvitamin D3, epi-25-Hydroxyvitamin D3, and 25-Hydroxyvitamin D2 in Plasma for Clinical Research Using High-Resolution Mass Spectrometry

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Keywords
Q Exactive Focus, UltiMate 3000, SOLAμ, Hypersil GOLD, SPE, micro-elution, vitamin D, clinical research, quantitation

Goal
To quantify 25-hydroxyvitamin D3 and epi-25-hydroxyvitamin D3 along with 25-hydroxyvitamin D2 in human plasma for clinical research using high-resolution mass spectrometry.

Introduction
Chromatographic separation is required for simultaneous analysis of 25-hydroxyvitamin D3 (25OHD3) and its epimeric form 3-epi-25-hydroxyvitamin D3 (epi-25OHD3) in a liquid chromatography-mass spectrometry (LC-MS) method because both compounds have the same molecular formulas and the same fragmentation spectra. We developed an LC method that provides baseline separation between the two isobaric compounds. We coupled this chromatographic method to an MS method, which collects high-resolution MS/MS spectra for compound identification and quantitation.

Experimental
Sample Preparation
Samples were processed by protein precipitation followed by solid phase extraction using Thermo Scientific™ SOLAμ™ HRP (2 mg) plates (P/N 60209-001). These fritless SPE plates deliver robust processing at elution volumes as low as 25 µL, thus eliminating the sample evaporation step and allowing for high-efficiency, cost-efficient analytical methods.

Briefly, 200 µL of methanol containing internal standards (D_6-25OHD3, D_3-epi-25OHD3) was added to 100 µL of sample (calibrator, control, or unknown). The resulting mixture was vortexed and centrifuged, and the supernatant was transferred to the well of the extraction plate containing 300 µL of water. The SPE wells were washed with 200 µL of water and 300 µL of 40% methanol. Analytes were eluted with 150 µL of methanol and further diluted with 100 µL of water.

Calibration Standards
Calibration standards at concentrations of 1, 2, 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 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 6, 15, and 50 ng/mL into previously analyzed pooled donor plasma.

Liquid Chromatography
An isocratic chromatographic separation was performed on a Thermo Scientific™ Dionex™ UltiMate™ 3000RS™ LC system with an 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™ PFP, 1.9 µm, 100 × 2.1 mm column (P/N 25402-202130). The total run time was five minutes.

Table 1. Concentrations of 25-hydroxyvitamin D2, 25-hydroxyvitamin D3 and epi-25-hydroxyvitamin D3 in in-house-prepared QC samples.

Analyte	QC0	QC1	QC2	QC3
	Concentration (ng/mL)			
250HD2	0.0	6.0	15.0	50.0
250HD3	8.4	14.4	23.4	58.4
epi-250HD3	0.6	6.6	15.6	50.6

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Mass Spectrometry

Compounds were detected on a Thermo Scientific™ 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 HCD cell using an optimized, compound-specific collision energy. The resulting MS/MS product ion spectrum is detected in the Thermo Scientific™ Orbitrap™ analyzer at a resolution of 35,000 (FWHM at *m/z* 200).

Method Evaluation

The limit of quantitation (LOQ) and linearity range were evaluated by collecting quintuplicate calibration curve data in three different batches.

Method precision and accuracy were evaluated by running a calibration curve and quintuplicate quality controls on three different days.

Matrix effects were evaluated by spiking 20 ng/mL of each analyte to eluent of processed plasma samples from six different donors and calculating recovery against the same concentration prepared in water.

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

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 1 ng/mL. Figure 1 shows combined stick mode chromatograms for internal standards and analytes at their respective LOQs illustrating over 15 scans collected across the peak.

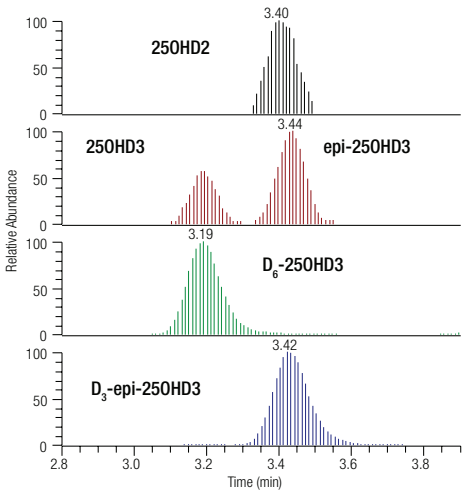

Figure 1. Chromatogram of the lowest calibration standard (1 ng/mL) reconstructed with mass accuracy of 5 ppm.

Calibration ranges were determined to be 1–100 ng/mL, where 100 ng/mL was the highest evaluated concentration. Figure 2 shows representative calibration curves for all analytes and chromatograms for the lowest calibration standard.

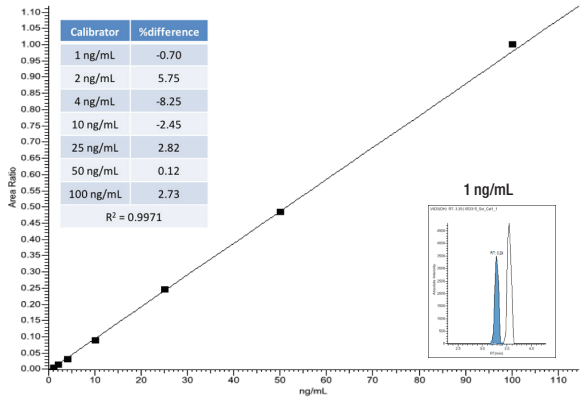

Figure 2a. Representative calibration curve for 25-hydroxyvitamin D3.

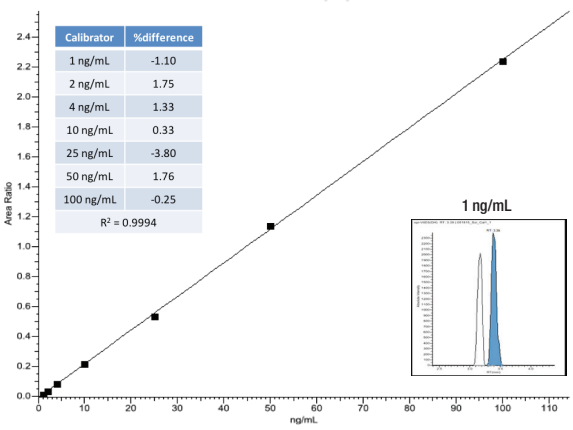

Figure 2b. Representative calibration curve for epi-25-hydroxyvitamin D3.

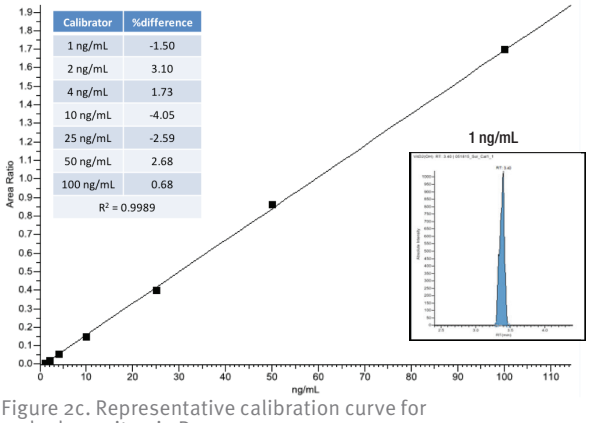

Figure 2c. Representative calibration curve for 25-hydroxyvitamin D2.

Inter-assay precision was better than 5.6% RSD, 5.1% RSD, and 7.7% RSD for 25OHD3, epi-25OHD3, and 25OHD2, respectively (Table 2).

Inter-assay precision was 6.4% RSD, 4.4% RSD, and 5.3% RSD for 25OHD3, epi-25OHD3, and 25OHD2, respectively (Table 3).

Figure 3 shows chromatograms of QC samples prepared in pooled donor plasma.

Method accuracy and matrix equivalence, as determined by analysis of NIST calibrators, ranged from 104% to 110% and from 90.7% to 114% for 25OHD3 and epi-25OHD3, respectively. For 25OHD2, the accuracy of the single calibration concentration was 97% (Table 4).

Table 2. Inter-assay precision for QC samples.

Analyte	QC0	QC1	QC2	QC3
	%RSD			
25OHD2	—	1.9–7.7	2.5–3.5	2.1–3.3
25OHD3	3.1–5.6	2.6–3.8	2.3–3.8	1.9–4.3
epi-25OHD3	—	2.2–5.1	2.3–4.1	1.3–3.6

Table 3. Inter-assay precision for QC samples.

Analyte	QC0	QC1	QC2	QC3
	%RSD			
25OHD2	—	5.3	3.1	4.2
25OHD3	6.4	4.1	3.2	3.5
epi-25OHD3	—	4.4	2.9	2.5

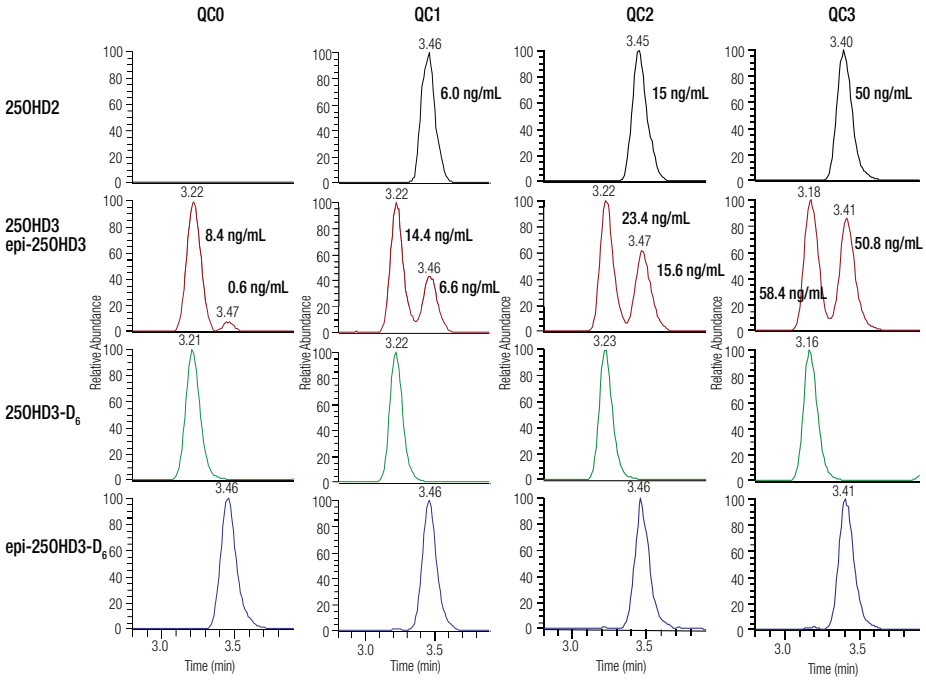

Figure 3. Chromatograms of QC samples reconstructed with mass accuracy of 5 ppm.

Table 4. Recovery of NIST calibrators calculated against a calibration curve prepared in ethanol showing matrix equivalence and method accuracy.

	25OHD3			epi-25OHD3			25OHD2		
	Expected (ng/mL)	Obtained (ng/mL)	% Recovery	Expected (ng/mL)	Obtained (ng/mL)	% Recovery	Expected (ng/mL)	Obtained (ng/mL)	% Recovery
Level 1	28.8	31.0	108	1.84	1.89	103	—	—	—
Level 2	18.1	19.9	110	1.29	1.17	90.7	—	—	—
Level 3	19.8	20.6	104	1.18	1.35	114	13.3	12.9	97.0
Level 4	29.4	31.2	106	26.4	24.0	90.9	—	—	—

Limited matrix effects were observed. Absolute %Recovery in six donor samples ranged from 77.6% to 104%, from 82.6% to 89.8%, and from 85.0% to 94.6% for 25OHD3, epi-25OHD3, and 25OHD2, respectively. Relative to the internal standard, %Recoveries ranged from 93.5% to 113%, from 105% to 117%, and from 101% to 114% for 25OHD3, epi-25OHD3, and 25OHD2, respectively.

Conclusion

We demonstrated a simple, high-efficiency method for analysis of 25-hydroxyvitamin D3, its epimeric form 3-epi-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. Method throughput can be doubled with a dual-channel Thermo Scientific™ Transcend™ II LC system (Figure 4).

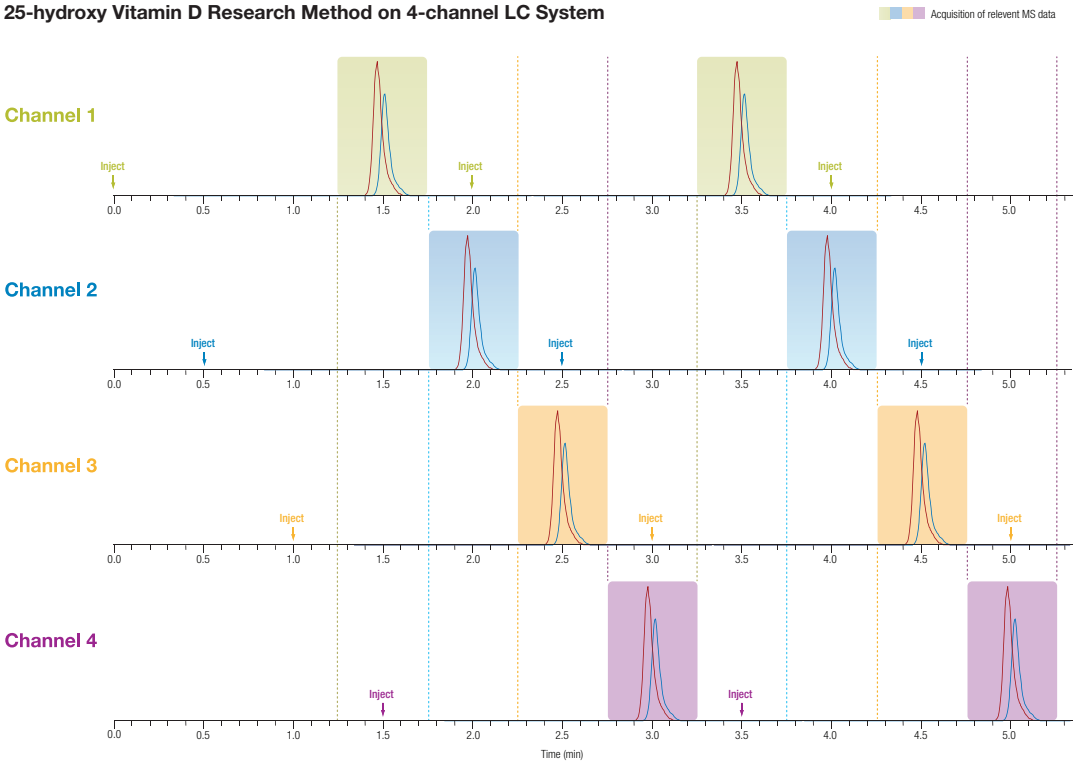

Figure 4. Execution of the method on a four-channel LC system.

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