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Evaluation of Bench-top Quadrupole Orbitrap Ultra High Resolution MS for Use in Clinical Research in Rapid Quantitative Analysis of Vitamin D in Human Plasma

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Purpose

To evaluate a rapid quantitative analysis of Vitamin D in human plasma samples using a bench-top quadrupole orbitrap high resolution mass spectrometer for use in clinical research.

Introduction

Clinical researchers commonly use a triple quadrupole mass spectrometer for analysis of 25-hydroxyvitamin D2 (25OHD2) and 25-hydroxyvitamin D3 (25OHD3) in plasma. We evaluated the Thermo Scientific™ Q Exactive™ Focus mass spectrometer equipped with Thermo Scientific™ Orbitrap™ technology using a fast, cost-efficient method that collected high resolution MS/MS spectra for improved method specificity. Protein precipitated plasma samples were analyzed with 3 min LC method, and high resolution MS/MS spectra were collected for each analyte. The most abundant fragment, besides water-loss, in each MS2 spectrum was selected for quantitative analysis (Table 3, Figure 1). The linearity range was 4-100 ng/mL (Figure 3, Figure 4); precision and accuracy were within 15%; matrix effects and interferences were not observed.

Instruments

LC system
Pump: Thermo Scientific™ Dionex™ Ultimate™ 3000
Autosampler: Thermo Scientific™ OAS-3X00TXRS
Q Exactive Focus orbitrap mass spectrometer

Methods

Calibrators

25-hydroxyvitamin D2 (25OHD2), 25-hydroxyvitamin D3 (25OHD3) were purchased from Cerillant Corporation. Since analyte free matrix was not available, calibration standards were prepared in ethanol. NIST controls and spiked plasma recovery data were used to prove calibration curve accuracy.

Sample Preparation

1.100 µL of plasma sample + 300 uL of acetonitrile containing internal standard (25 ng/mL d6-25-hydroxyvitamin D3).
2.Vortex, centrifuge
3.Inject 50 µL into LC-MS system.

LC method

Mobile phase A: 0.1% formic acid in water
Mobile phase B: 0.1% formic acid in methanol
Column: Thermo Scientific™ Hypersil GOLD™ aQ 5µ 50x2.1mm

TABLE 1. HPLC Gradient Method

Time (min)	Flow rate (mL/min)	%A (mobile phase)
0	0.70	20
0.2	0.70	20
0.3	0.70	5
1.5	0.70	5
1.6	0.70	20
2.0	0.70	20

Mass Spectrometry Method

APCI ionization source

Data acquisition method: parallel reaction monitoring (PRM) experiment collecting MS2 spectra for each analyte.

Resolution: 17.5K

Data Analysis

Thermo Scientific™ TraceFinder 3.2 software was used for all data processing and analysis.

Method Performance Evaluation

QC samples were prepared by spiking previously analyzed donor plasma to concentrations specified in Table 2 and Figure 2.

TABLE 2. QC Samples Concentrations

Analyte/Concentration ng/mL	QC1	QC2	QC3
25-Hydroxyvitamin D2	10.0	20.0	30.0
25-Hydroxyvitamin D3	25.1	39.9	60.8

Method accuracy was evaluated by obtaining %recovery for NIST controls.

Method precision was evaluated by analyzing 5 replicates of each QC sample in 3 different days (Table 4, Table 5).

Matrix effects were evaluated by spiking previously analyzed plasma from 5 different donors with 25OHD3 and 25OHD2 concentrations of 15 ng/mL and calculating % RSD and % recovery from 5 replicate analysis (Table 7).

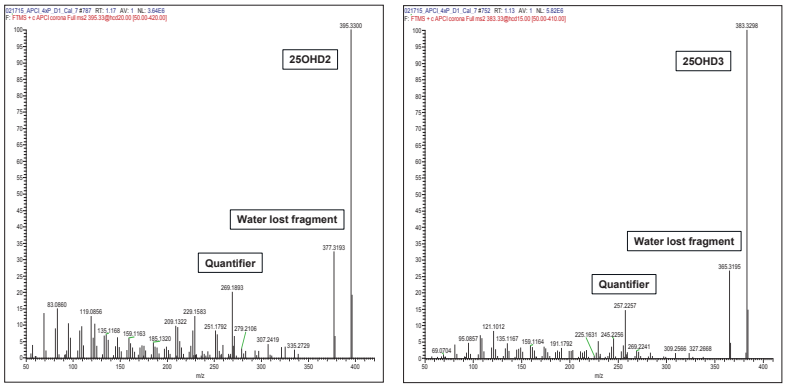
System reproducibility was evaluated by analyzing 5 replicates of each calibration standard (Table 6).

TABLE 3. PRM transitions of targets and internal standards

Compound	Precursor (m/z)	Quantitation Fragment (m/z)	Collision Energy (V)	Polarity
25OHD2	395.3308	269.1898	20	Positive
25OHD3	383.3308	257.2264	15	Positive
D6-25OHD3 (IS)	389.3685	263.2638	20	Positive

Results

FIGURE 1. 25OHD2, 25OHD3 MS/MS Spectrum



Collision energy (20 eV) was optimized to maximize response of quantifying fragment ion (m/z=269.1893)

Collision energy (15 eV) was optimized to maximize response of quantifying fragment ion (m/z=257.2257)

FIGURE 2. Chromatogram of QC Plasma Samples

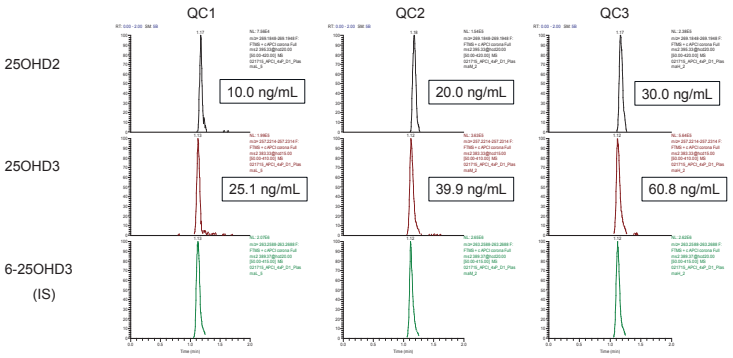


FIGURE 3. Calibration Curves with Lowest Calibration Standard Peaks

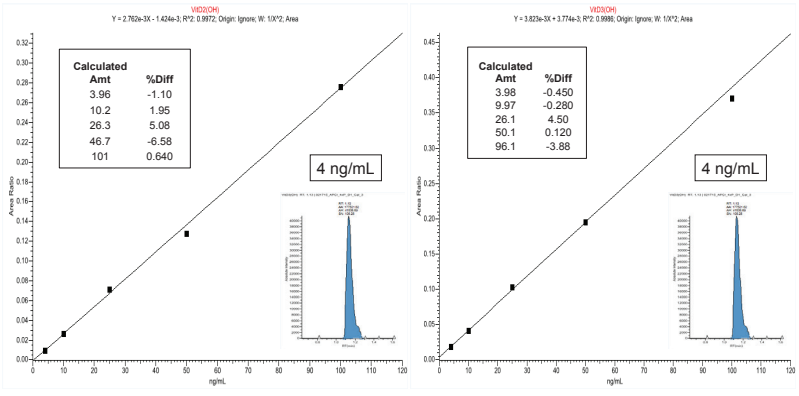
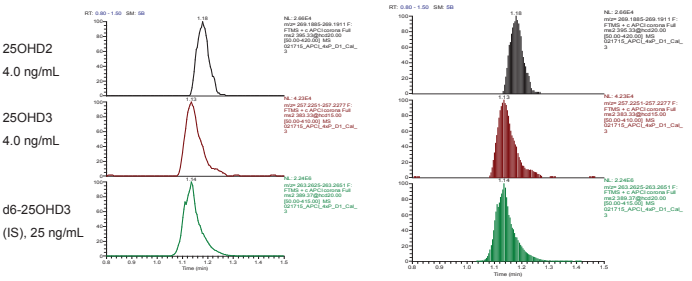


FIGURE 4. Chromatogram of Lowest Calibration Standard



Chromatogram reconstructed with mass accuracy of 5 ppm

TABLE 4. Intra-assay Precision and Accuracy *

Analyte	QC1	QC2	QC3
		%RSD	
25-Hydroxyvitamin D2	6.30 - 12.7	5.33 - 13.0	2.77 - 5.30
25-Hydroxyvitamin D3	3.21 - 7.64	2.64 - 5.89	3.70 - 4.70
		%Recovery	
25-Hydroxyvitamin D2	87.0 - 100	99.9 - 109	102 - 106
25-Hydroxyvitamin D3	82.9 - 83.5	94.5 - 96.3	91.1 - 101

* 5 replicates of QC samples containing 25OHD2 and 25OHD3 were proceed and analyzed in 3 batches

TABLE 5. Inter-assay Precision and Accuracy *

Analyte	QC1	QC2	QC3
		%RSD	
25-Hydroxyvitamin D2	10.5	9.01	4.51
25-Hydroxyvitamin D3	5.56	3.93	6.1
		%Recovery	
25-Hydroxyvitamin D2	93.2	105	104
25-Hydroxyvitamin D3	82.9	95.1	97.3

* 5 replicates of QC samples containing 25OHD2 and 25OHD3 were proceed and analyzed in 3 batches

TABLE 6. System Reproducibility *

Analyte	Cal-4	Cal-10	Cal-25	Cal-50	Cal-100
			%RSD		
25OHD2	1.60	2.52	4.27	1.65	2.71
25OHD3	4.72	1.37	4.77	2.01	3.86
			%Recovery		
25OHD2	99.7	101	96.8	105	96.9
25OHD3	100	101	97.0	98.8	103

* 5 replicate injections of each calibration standard containing 25OHD2 and 25OHD3 were performed to demonstrate the system reproducibility

TABLE 7. Matrix Effects *

Analyte	Plasma-1	Plasma-2	Plasma-3	Plasma-4	Plasma-5
			%RSD		
25OHD2	3.65	5.36	9.17	5.90	10.6
25OHD3	2.70	3.48	1.09	4.31	6.99
			%Recovery		
25OHD2	77.2**	91.7	79.9**	86.5	85.3
25OHD3	112	104	106	109	111

* 15 ng/mL of each analyte was spiked in 5 different plasma samples in 5 replicates

** 25OHD2 % recovery will be increased when deuterated analog will be used as internal standard.

Conclusion

- Method meets clinical research requirements for analysis of Vitamin D 25-hydroxy metabolites in plasma samples.
- The calibration range used in method evaluation experiments was 1 to 100 ng/mL, but for improved method robustness, recommended concentration range is 4-100 ng/mL.
- Deuterated analog of 25OHD2 is recommended to correct for limited matrix effects we observed during method evaluation.

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