Analysis of 25-Hydroxyvitamin D in Serum Using an Automated Online Sample Preparation Technique with a High-Resolution Benchtop Orbitrap Mass Spectrometer

Matthew Berube, Joe DiBussolo, Catherine Lafontaine, Yang Shi
Thermo Fisher Scientific, Franklin, MA

Application Note 567

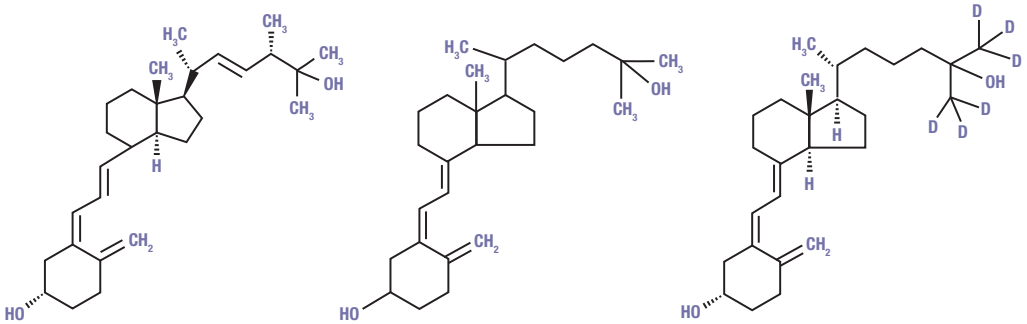
Key Words
Transcend TLX-1 System, TurboFlow Technology, Exactive Plus, Vitamin D

Goal
To demonstrate the effectiveness of a clinical research method for the quantitation of 25-hydroxyvitamin D using online sample preparation and high-resolution, accurate mass (HR/AM) quantitation with a Thermo Scientific Exactive Plus Orbitrap mass spectrometer.

Introduction
Blood levels of 25-hydroxyvitamin D_2 and 25-hydroxy-vitamin D_3 are commonly tested by clinical researchers to assess vitamin D sufficiency. In the last decade, liquid chromatography coupled with triple quadrupole mass spectrometry (LC-MS/MS) has become a popular technique for such measurements. Due to their higher resolving power relative to triple-stage quadrupole mass spectrometers, Orbitrap™-based mass spectrometers are better able to resolve analytes from sample matrices. In addition, the ease of initial method set up and daily use provides an advantage over triple-stage quadrupole mass spectrometers for clinical research.

A method has been created that allows precipitated serum to be injected into an HPLC system with minimal sample preparation and analyzed by an Exactive™ Plus benchtop Orbitrap mass spectrometer. Total method time is 7.75 minutes on a Thermo Scientific Transcend TLX-1 system utilizing TurboFlow technology. Throughput can be increased to a sample every 3.7 minutes by using a Transcend™ TLX-2 multiplexed UHPLC system or 1.9 minutes with a Transcend TLX-4 system.

Experimental
Standard solutions of 25-hydroxyvitamin D_2, 25-hydroxy-vitamin D_3, and deuterated 25-hydroxyvitamin D_3 internal standard were obtained from Cerilliant, Inc. (Figure 1). Six calibrators at 2, 5, 10, 25, 50 and 100 ng/mL and three QCs at 5, 40 and 80 ng/mL were prepared by fortifying bovine serum albumin diluent with 200 ng/mL 25-hydryoxyvitamin D_2 and D_3 standard mix. Precipitating reagent was prepared by adding deuterated D_6-25-hydroxy-vitamin D_3 to acetonitrile for a final concentration of 75 ng/mL. In addition, pooled human serum samples were crashed 2 to 1 with acetonitrile and spiked with analytes for a final concentration of 20 ng/mL for 25-hydroxy-vitamin D_2 and 25-hydroxyvitamin D_3, and 50 ng/mL of D_6 25-hydroxyvitamin D_3 internal standard.



25-Hydroxyvitamin D_2
$C_{28}H_{44}O_2$ (MW 412.65)

25-Hydroxyvitamin D_3
$C_{27}H_{44}O_2$ (MW 400.63)

D_6-25-Hydroxyvitamin D_3
$C_{27}H_{38}D_6O_2$ (MW 406.67)

Figure 1. Analytes

Thermo SCIENTIFIC

Samples were prepared by adding 200 µL of precipitating reagent containing internal standard to each centrifuge tube containing 100 µL of calibrants and controls. Tubes were vortexed for 30 seconds and then centrifuged at 5,000 RCF for 10 minutes. Supernatants were then aliquoted into autosampler vials for analysis. Calibration curves and QCs were run in triplicate each day across four days. In addition, 800 pooled serum sample replicates containing 20 ng/mL 25-hydroxyvitamin D_2 and 25-hydroxyvitamin D_3 and 50 ng/mL of D_6-25-hydroxy-vitamin D_3 internal standard were injected to test robustness of the method. Thermo Scientific Xcalibur software was used to collect data and analyze the results. The Exactive Plus mass spectrometer was used with an APCI source in positive ionization mode. Full-scan data was collected from *m/z* 350 to 425.

LC/MS Conditions

TurboFlow Method Parameters (see also Figure 2)	
Plumbing mode:	Focus Mode
Column:	Thermo Scientific TurboFlow XL C-18P 0.5 x 50 mm
Injection volume:	50 µL
Solvent A:	0.1% formic acid in water
Solvent B:	0.1% formic acid in methanol
Solvent C:	40:40:20 acetonitrile: isopropyl alcohol: acetone (v:v:v)
Analysis time:	7.75 minutes
Cycle time when multiplexed 4x:	1.9 minutes

HPLC Method Parameters	
Analytical column:	Thermo Scientific Accucore C18 3 x 50 mm 2.6 µm
Solvent A:	0.1% formic acid in water
Solvent B:	0.1% formic acid in methanol

Mass Spectrometer Parameters	
Scan mode:	Full
Scan range:	*m/z* 350 – 425
Fragmentation:	None
Polarity:	Positive
Microscans:	1
Resolution:	70,000
AGC target:	3 x 10^6
Maximum inject time:	200

Ion Source Parameters	
Ion source:	APCI
Discharge current:	3.5 uA
Vaporizer temperature:	500 °C
Sheath gas pressure:	30 units
Ion sweep gas pressure:	1 unit
Aux gas pressure:	5 units
Capillary temperature:	250 °C
S-Lens RF level:	60

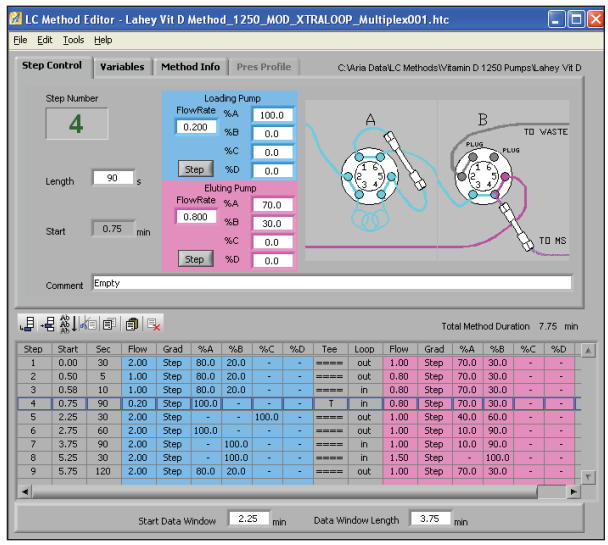


Figure 2. TurboFlow method details

Results and Discussion

The lower limit of quantitation (LLOQ) was determined to be 2 ng/mL for both analytes in BSA as indicated in Figure 3. Limits of quantitation (LOQs) were estimated from the triplicate injections of the standard solutions. The signal-to-noise ratio was greater than 10 and the coefficient of variation (CV) values were less than 10% at the LLOQ of 2 ng/mL for both 25-hydroxyvitamin D_2 and 25-hydroxyvitamin D_3 (Table 1). The correlation coefficients obtained using 1/X weighted linear regression analysis of the standard curves were greater than 0.99 for both analytes (Figures 4 and 5). A relative standard deviation (%RSD) test was performed in pooled human serum fortified with analytes at 20 ng/mL and crashed with internal standard solution for a total internal standard concentration of 50 ng/mL. The RSDs of ten replicate injections were less than 10% for both analytes (Table 2). A recovery study was also performed using a neat standard of 20 ng/mL 25-hydroxyvitamin D_2 and 25-hydroxyvitamin D_3 with 50 ng/mL D_6-25-hydroxy-vitamin D_3. The standard was injected ten times on the TurboFlow™ column and analytical column, and ten times on the analytical column only, and area counts were compared. The relative recoveries were 97% and 99% for 25-hydroxyvitamin D_2 and 25-hydroxyvitamin D_3, respectively.

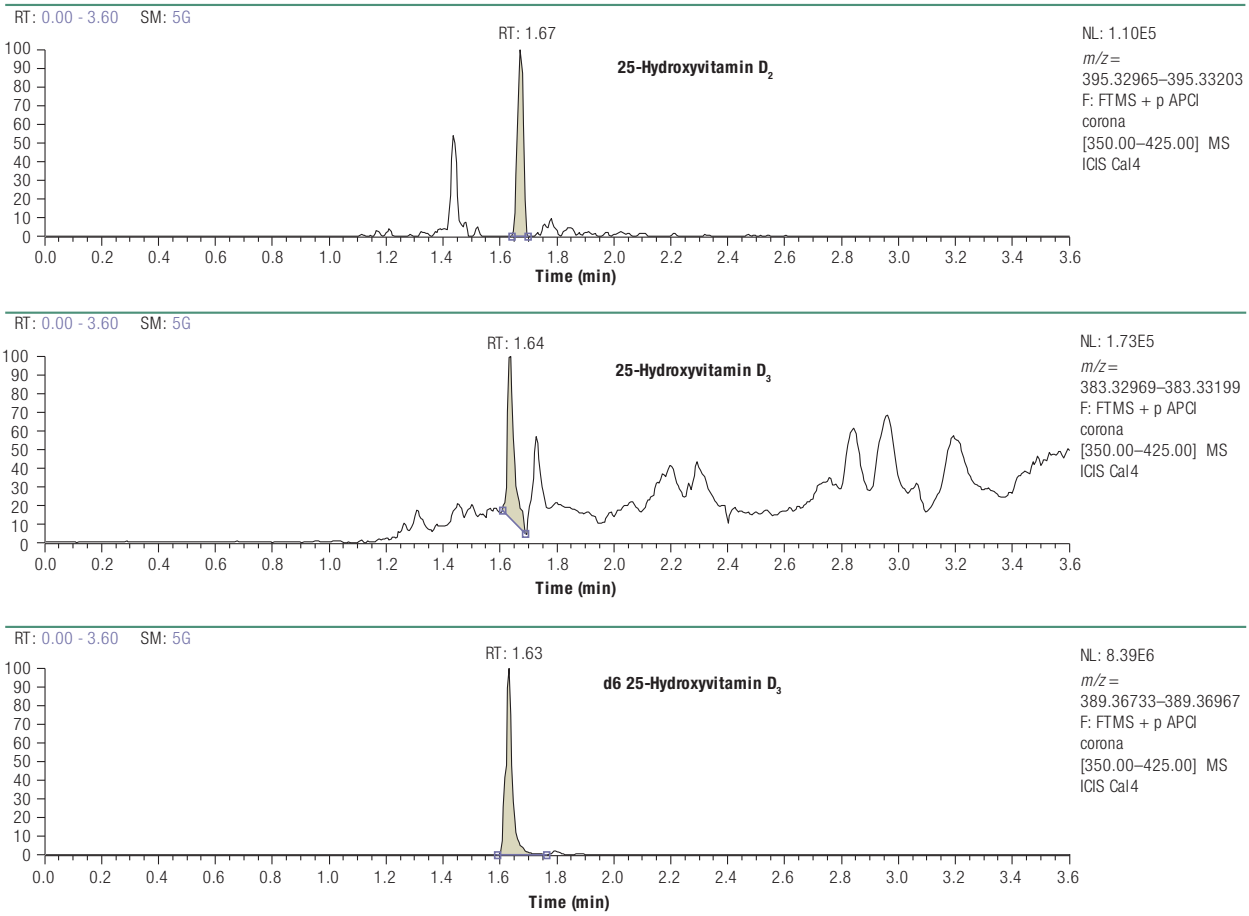


Figure 3. Chromatograms at LLOQ of 2 ng/mL with 50 ng/mL internal standard

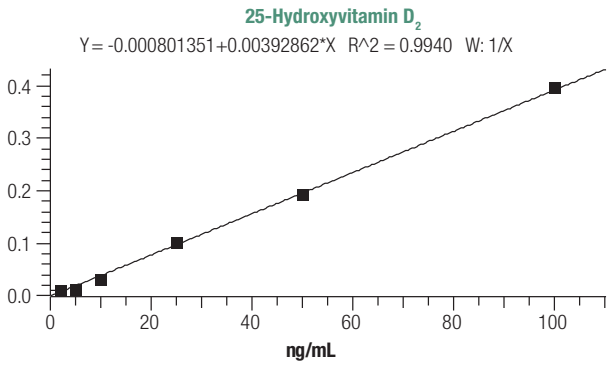


Figure 4. Calibration curve of 25-hydroxyvitamin D_2 in BSA

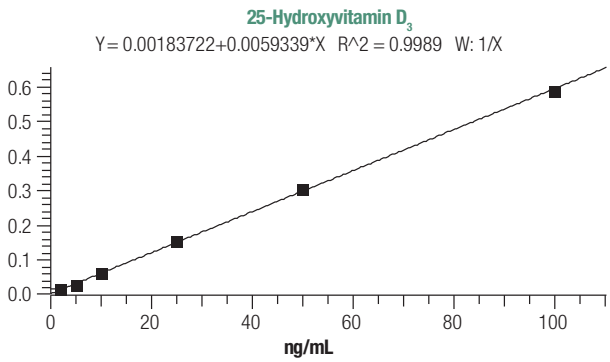


Figure 5. Calibration curve of 25-hydroxyvitamin D_3 in BSA

Table 1. 2 ng/mL replicate LLOQ injections

25-hydroxyvitamin D_2	2 ng Area
Replicate 1	134195
Replicate 2	162585
Replicate 3	148309
Mean	148363
SD	14195.1
%CV	9.6

25-hydroxyvitamin D_3	2 ng Area
Replicate 1	201766
Replicate 2	242186
Replicate 3	212094
Mean	218682
SD	20999.9
%CV	9.6

Table 2. 20 ng/mL serum injection replicates

D_2 20 ng Serum	Area
Replicate 1	4464244
Replicate 2	3757594
Replicate 3	4544819
Replicate 4	4332109
Replicate 5	3857037
Replicate 6	4581097
Replicate 7	5148234
Replicate 8	4704084
Replicate 9	4319873
Replicate 10	4175023
Mean	4388411
SD	405245.1
%CV	9.2

D_3 20 ng Serum	Area
Replicate 1	11759664
Replicate 2	10759647
Replicate 3	10886536
Replicate 4	10825748
Replicate 5	12543252
Replicate 6	12223745
Replicate 7	11278373
Replicate 8	11445949
Replicate 9	12537176
Replicate 10	11033701
Mean	11529379
SD	698829.3
%CV	6.1

Conclusion

An Exactive Plus high-resolution Orbitrap mass spectrometer with TurboFlow automated on-line sample extraction technology provides reliable detection for clinical researchers of 25-hydroxyvitamin D_2 and 25-hydroxyvitamin D_3 in serum.

In addition, the Exactive Plus MS offers higher resolving power and easier initial method setup than triple quadrupole mass spectrometers. Throughput can be increased to a sample every 3.7 minutes by using a Transcend TLX-2 multiplexed UHPLC system or a sample every 1.9 minutes with a Transcend TLX-4 system.

For Research Use Only. Not for use in diagnostic procedures.

ISO 9001 REGISTERED COMPANY
Thermo Fisher Scientific, San Jose, CA USA is ISO Certified.

Certified System ISO 9001
Thermo Fisher Scientific (Bremen) GmbH Management System Registered to ISO 9001:2008

www.thermoscientific.com

Africa-Other +27 11 570 1840
Australia +61 2 8844 9500
Austria +43 1 333 50 34 0
Belgium +32 53 73 42 41
Canada +1 800 530 8447
China +86 10 8419 3588
Denmark +45 70 23 62 60
Europe-Other +43 1 333 50 34 0
Finland /Norway/Sweden +46 8 556 468 00
France +33 1 60 92 48 00
Germany +49 6103 408 1014
India +91 22 6742 9434
Italy +39 02 950 591
Japan +81 45 453 9100
Latin America +1 608 276 5659
Middle East +43 1 333 50 34 0
Netherlands +31 76 579 55 55
South Africa +27 11 570 1840
Spain +34 914 845 965
Switzerland +41 61 716 77 00
UK +44 1442 233555
USA +1 800 532 4752

Thermo SCIENTIFIC
Part of Thermo Fisher Scientific

AN63534_E 06/12S