Application Note: 396

Quantitative Analysis of 25-OH-D_2 and 25-OH-D_3 in Serum by LC-MS/MS

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Key Words

- TSQ Quantum Access™
- Accela™ LC System
- Quantitation
- Vitamin D

Introduction

Vitamin D is a group of fat-soluble prohormones that helps the body absorb calcium and maintain proper levels of calcium and phosphorus in the blood. A vitamin D deficiency results in impaired bone mineralization and leads to bone softening diseases, such as rickets in children and osteomalacia and osteoporosis in adults, as well as autoimmune and nervous system diseases. A vitamin D overdose can raise blood levels of calcium and cause gastrointestinal symptoms and kidney disease. High blood levels of calcium also can cause heart rhythm abnormalities and calcinosis, the deposition of calcium and phosphate in the body's soft tissues.

Vitamin D exists naturally in two major forms: vitamin D_2 and vitamin D_3. (See Figure 1.) Vitamin D_2 is derived from plant and fungal sources. Vitamin D_3 is derived from animal sources and is produced in skin exposed to ultraviolet (UVB) radiation from the sun. In the body, vitamin D is metabolized to 25-hydroxy-vitamin D (25-OH-D) in the liver and subsequently to biologically active 1,25-dihydroxy-vitamin D ($1,25(OH)_2D$) in the kidneys.

The level of vitamin D in serum is measured by analyzing its major metabolites. Among the vitamin D metabolites, 25-OH-D has the highest concentration and longest half-life in blood. It is considered to be the best indicator of vitamin D concentration in the blood. Serum 25-OH-D concentrations in the range 32–80 ng/mL (80–200 nMol/L) are optimum. Concentrations below 32 ng/mL indicate a vitamin D deficiency, and concentrations above 100 ng/mL are regarded as toxic.

Goal

To develop a quantitative, efficient LC-MS method for analysis of 25-hydroxy-vitamin D_3 and 25-hydroxy-vitamin D_2 in serum.

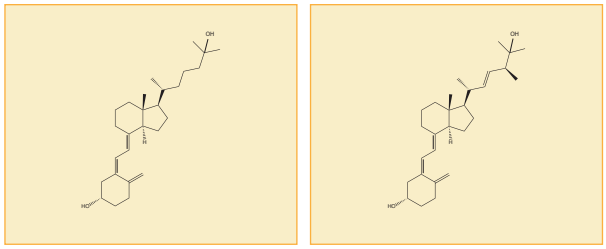

Figure 1: 25-hydroxy-vitamin-D_3 and 25-hydroxy-vitamin-D_2 chemical structures.

Experimental Conditions

Figure 2 illustrates the workflow for the quantitative analysis of 25-OH-D_3 and 25-OH-D_2 in serum.

Chemicals and Reagents

The reagents 25-OH-D_3 and 25-OH-D_2 were purchased from Sigma-Aldrich (Sigma-Aldrich, St. Louis, MO) and were stored at –20 °C. The internal standard [$^2H_6$]-25-OH-D_3 was purchased from Medical Isotopes (Medical Isotopes, Inc., Pelham, NH) and was stored at –70 °C. Bovine serum albumin and PBS buffer were purchased from Sigma-Aldrich and were stored in a refrigerator. Bovine serum was used because human serum with low 25-OH-D_3 and 25-OH-D_2 content was not commercially available.

Sample Preparation

To prepare each sample for analysis, 100 µL of internal standard solution (100 ng/mL [$^2H_6$]-25-OH-D_3 in ethanol) and 1 mL of acetonitrile were added to 250 µL of serum. The sample was vortexed and centrifuged. The supernatant was decanted and evaporated to dryness at 37 °C. The sample was then reconstituted in 100 µL of methanol. After reconstitution, 25 µL was injected into the LC-MS.

Calibration Curve Standards Preparation

A standard stock solution of 100 µg/mL 25-OH-D_3 and 25-OH-D_2 in ethanol was prepared. Standard spiking solutions of 25-OH-D_3 and 25-OH-D_2 in ethanol at concentrations of 10 µg/mL, 1 µg/mL, and 100 ng/mL were prepared by serial dilution. The appropriate amount of the standard spiking solution was added to 250 µL of 6% BSA in 0.01M PBS (pH 7.4) to prepare calibration standards at the following eight concentrations: 4 ng/mL, 10 ng/mL, 20 ng/mL, 40 ng/mL, 100 ng/mL, 200 ng/mL, 400 ng/mL, and 500 ng/mL. The standards were processed

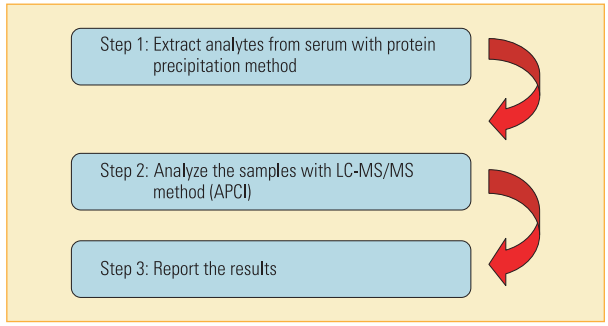

Figure 2: Workflow for the quantitative analysis of 25-OH-D_3 and 25-OH-D_2 in serum.

with the sample preparation procedure described above. The standard stock solution and the standard spiking solutions were stored at –20 °C.

HPLC

HPLC analysis was performed using the Accela High Speed LC System (Thermo Fisher Scientific, San Jose, CA). The 25 µL samples were injected directly onto a 2.1 mm ID×50 mm column, packed with 5 µm, C18-aQ stationary phase. Mobile phase A was water containing 0.1% formic acid. Mobile phase B was methanol containing 0.1% formic acid. The flow rate was 400 µL/min. The gradient is described in Table 1.

Time (min)	% A	% B
0	40	60
0.5	40	60
1.5	2	98
2.5	2	98
2.6	40	60
3.5	40	60

Table 1: Gradient profile

MS

MS analysis was carried out on a TSQ Quantum Access triple stage quadrupole mass spectrometer with an Ion Max™ ion source and an atmospheric pressure chemical ionization (APCI) probe (Thermo Scientific, San Jose, CA). The MS conditions were as follows:

Ion polarity: Positive ion mode
Vaporizer temperature: 375 °C
Capillary temperature: 250 °C
Discharge current: 2.0 µA
Sheath gas pressure (N_2): 35 units
Auxiliary gas pressure (N_2): 5 units
Scan type: H-SRM
Scan time: 0.050 s

Table 2 lists the SRM transitions and their parameters. The parent molecular ions for the three analytes show a significant ion due to the loss of a water molecule from the molecular ion. This allows the use of the MH-18 *m/z* peak for quantitation by SRM.

Analyte	Parent Mass	Product Mass	Collision Energy	Tube Lens
25-OH-D_3	383.28*	211.094	21	104
25-OH-D_2	395.29*	209.039	23	102
25-OH-D_3 (Internal Standard)	389.341*	211.157	31	103

*parent molecular ion minus water [MH-18]

Table 2: SRM transitions

Results and Discussion

Representative - SRM chromatograms for 25-OH-D_3 and 25-OH-D_2 at 4 ng/mL and 100 ng/mL are shown in Figures 3 and 4, respectively. Clearly identifiable and quantifiable peaks were observed.

Figures 5 and 6 show the linear fit calibration curves for 25-OH-D_3 and 25-OH-D_2, respectively. The calibration curves have R^2 values greater than 0.999, which indicate excellent linear fits over the dynamic range of 4–500 ng/mL for 25-OH-D_3 and 2–500 ng/mL for 25-OH-D_2. The LOQ value is 4 ng/mL for 25-OH-D_3 and 2 ng/mL for 25-OH-D_2, with LOD values ~4 times lower for each analyte.

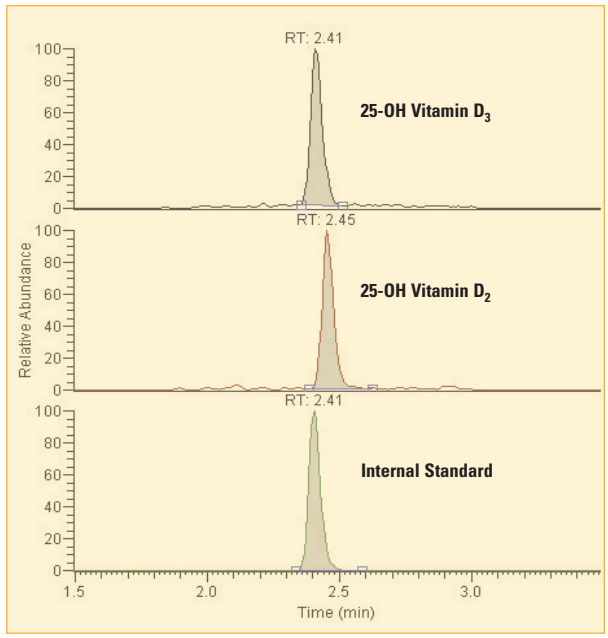

Figure 3: Chromatograms of 4 ng/mL standard

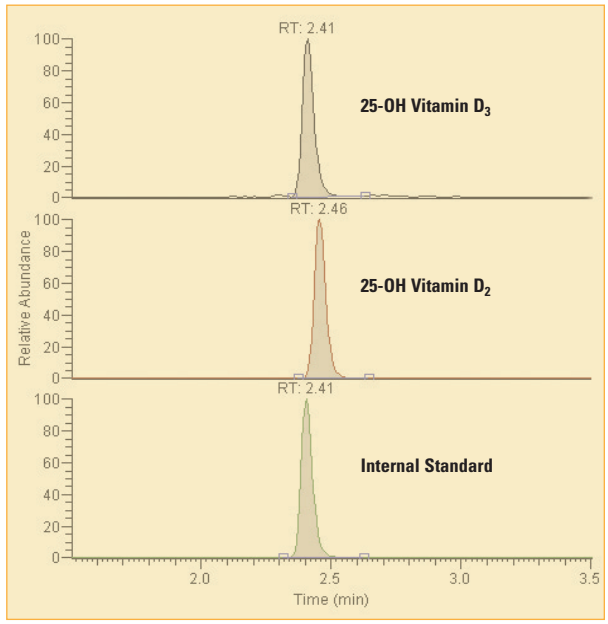

Figure 4: Chromatograms of 100 ng/mL standard

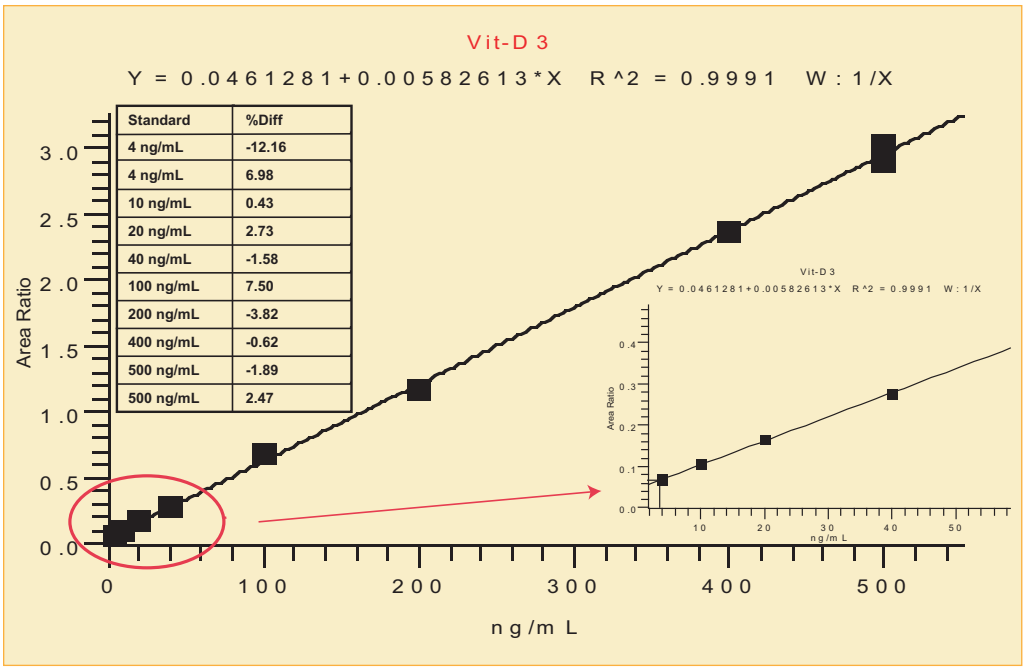

Figure 5: 25-OH-D_3 calibration curve

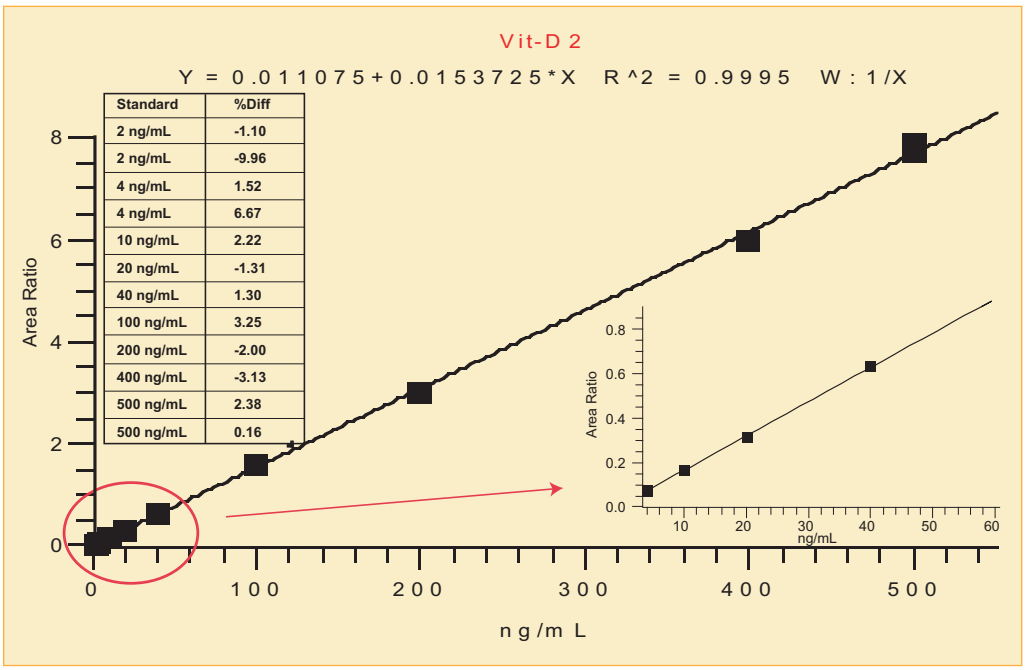

Figure 6: 25-OH-D_2 calibration curve

The method accuracy and precision were evaluated by analyzing quality control samples prepared at concentrations of 10 ng/mL, 100 ng/mL, and 400 ng/mL in 6% BSA. Intra-assay variability was determined by processing and analyzing five replicates of each QC sample. Inter-assay variability was determined by processing and analyzing five replicates of each QC sample in three different batches. Intra-assay and inter-assay variability are <8.7% and <5.8% for 25-OH-D_3, and <3.2% and <2.5% for 25-OH-D_2, respectively, meeting industry criteria.

Conclusion

A fast, sensitive and reliable LC-MS/MS-SRM method has been developed for the determination of 25-OH D_2 and 25-OH-D_3 in serum. Sample analysis was performed with a runtime of less than four minutes with a quantification limit of 4 ng/mL (10 nMol/L) for 25-OH-D_3, 2 ng/mL (4.85 nMol/L) for 25-OH-D_2 and a linearity range of 4–500 ng/mL (10–1250 nMol/L) for 25-OH D_3, 2–500 ng/mL (4.85–1212.7 nMol/L) for 25-OH-D_2. Data was acquired using H-SRM. The low intra-assay and inter-assay variability of the results demonstrates the reliability of the method.

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