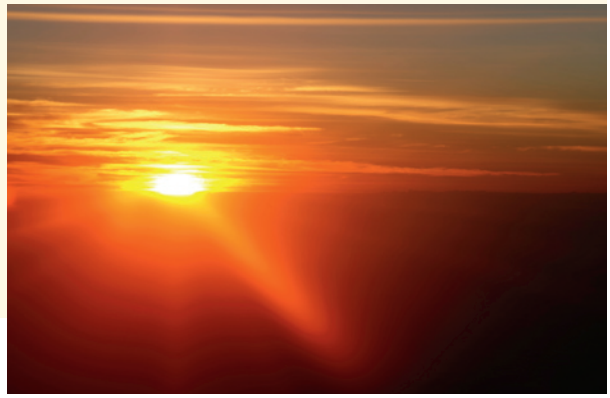
APPLICATION BRIEF

Clinical

# Analysis of 25-Hydroxyvitamin $D_2$ and $D_3$ in Serum Using UHPLC/SQ MS



Vitamin D (where D refers to $D_2$ or $D_3$) is a fat-soluble pro-hormone that when metabolized into its active form targets less than 200 human genes in a wide variety of tissues in the body. In addition to the major physiological function of vitamin D metabolites to maintain calcium and phosphate homeostasis, vitamin D status has been associated with a variety of disease states including cancer, cardiovascular disease, diabetes, multiple sclerosis, osteoporosis, rheumatoid arthritis, and chronic pain.

Vitamin $D_3$ (cholecalciferol) is formed in the skin upon exposure to sunlight and vitamin $D_2$ (ergocalciferol) is obtained from the ultraviolet irradiation of plant materials (Figure 1). Natural sources of vitamin $D_3$ include oily fish (such as salmon or mackerel), cod liver oil, and fortified food (such as milk, orange juice, butter, cheeses, and breakfast cereals). Prescription vitamin D preparations contain ergocalciferol (50,000 IU/capsule) while over-the-counter supplements contain cholecalciferol (400, 800, 1000, and 2000 IU/capsule).

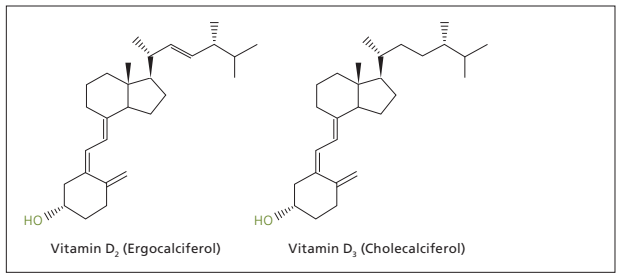



*Figure 1.* Structures of Vitamin $D_2$ (ergocalciferol) and Vitamin $D_3$ (cholecalciferol).

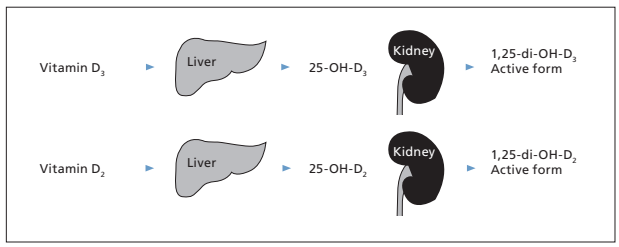



*Figure 2.* Formed in the skin, vitamins $D_3$ and $D_2$ are converted into their metabolically active forms by subsequent reactions in the liver and kidneys.

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Upon penetration of the skin with ultraviolet B radiation, vitamin $D_3$ is synthesized from 7-dehydroxycholesterol to form previtamin $D_3$ which is rapidly converted by a thermally-induced process to vitamin $D_3$. Vitamin $D_3$ is converted into its metabolically active form 1,25-dihydroxyvitamin $D_3$ ($1,25(OH)_2D_3$) by two subsequent hydroxylations, consisting of 25-hydroxylation in the liver followed by 1α-hydroxylation in the kidney (Figure 2). Both hydroxylations are carried out by specific cytochrome P450-containing enzymes, the first by vitamin D 25-hydroxylase (CYP27) and the second by 25-hydroxyvitamin D 1α-hydroxylase (CYP1α).

25-Hydroxyvitamin D is the metabolite measured in the blood to determine the vitamin D status of patients. Methodologies for measuring serum 25(OH)D include competitive protein binding assays (CPBA), radio-immunoassay (RIA), enzyme-linked immunoassay (ELISA), high-performance liquid chromatography (HPLC), liquid chromatography coupled with mass spectrometry (LC/MS), and random access automated assay (RAAA) based on chemiluminescence assay technology. Conventional techniques for vitamin D analysis, based on immunoassay or LC/UV, often lack adequate sensitivity, specificity, and speed; thus, interest in UHPLC/MS methods is growing. An advantage of UHPLC/MS technology is the ability to differentiate between $25(OH)D_2$ and $25(OH)D_3$ which is particularly useful when monitoring the vitamin D levels of patients being treated with vitamin $D_2$.

## Experimental Conditions

Target Analytes: 25-Hydroxyvitamin $D_2$ and $D_3$

### Sample Preparation Conditions

Human serum controls and patient unknown samples were obtained. 150 µL serum was taken and 50 µL internal standard, 25-Hydroxyvitamin $D_3$-$d_6$, added and vortex. 150 uL 0.2 M $ZnSo_4$ was added and again vortex. Finally, 750 mL Hexane was added to the mixture and centrifuged at 8000 RPM for 7 min. The top layer, organic phase, was removed and blown down under nitrogen and reconstituted in 100 µL MeOH/$H_2O$ and analyzed by HPLC/SQ MS.

### Liquid Chromatography Conditions

| | |
|---|---|
| Pump Type: | PerkinElmer® Flexar™ FX-10 |
| Column: | PerkinElmer Brownlee™ HRes Analytical DB C18 column (2.1 mm x 50 mm, 1.9 µm) |
| Mobile Phase: | A: water containing 2 mM Ammonium Acetate and 0.1% formic acid<br>B: methanol containing 2 mM Ammonium Acetate and 0.1% formic acid |
| Flow Rate: | 0.3 mL/min |
| Injection Volume: | 8 µL in micro liter pickup mode |

Isocratic Conditions:

| Time (min) | %A | %B |
|---|---|---|
| 0 | 7 | 93 |
| 6 | 7 | 93 |

### Mass Spectrometer Conditions

Ionization: Ultraspray™ ESI – Positive mode

The $[M+H]^+$ ions of each of the analytes were monitored:

Time Period 1: (0-6 min) SIM ions 401.4, 407.4, 413.4 were monitored for 25-Hydroxyvitamin $D_3$, 25-Hydroxyvitamin $D_3$-$d_6$ and 25-Hydroxyvitamin $D_2$ respectively; dwell time of 150 ms each

Capillary Exit Voltage: 45 V

## Results

UHPLC/SQ MS allows for increased specificity for detection as compared to competitive protein binding assays (CPBA), radio-immunoassay (RIA), enzyme-linked immunoassay (ELISA), high-performance liquid chromatography (HPLC) as it can monitor both $D_2$ and $D_3$ levels separately as opposed to total vitamin D. This is important especially for patients being treated with vitamin $D_2$.

Measurement of both 25-hydroxyvitamin $D_2$ and $D_3$ is possible using the Flexar SQ 300 MS with a simple protein precipitation/ LLE method. The conditions used here for routine vitamin D testing by UHPLC/SQ MS resulted in highly symmetric peaks with a run time of 6 min which is much faster than competing techniques. We were able to detect and quantify patient samples below 4 ng/mL.

According to current industry definitions, severe deficiency can be defined as <6 ng/mL, deficiency as 16 ng/mL, insufficiency as <20 ng/mL and as normal >20 ng/mL.[1]

Therefore, the Flexar SQ 300 MS is fully capable of detecting and quantifying within the clinical significant range for vitamin D.

[1]*Mosekilde L et al, Ugeskr Læger 167/1, 2005, 29-33.*

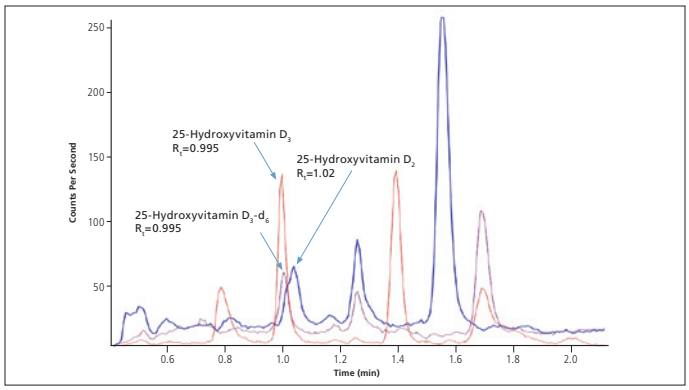


*Figure 3.* Patient sample with separation and detection of 25-hydroxyvitamin $D_2$ (blue trace), 25-hydroxyvitamin $D_3$ (red trace) and 25-hydroxyvitamin $D_3$-$d_6$ (purple trace).

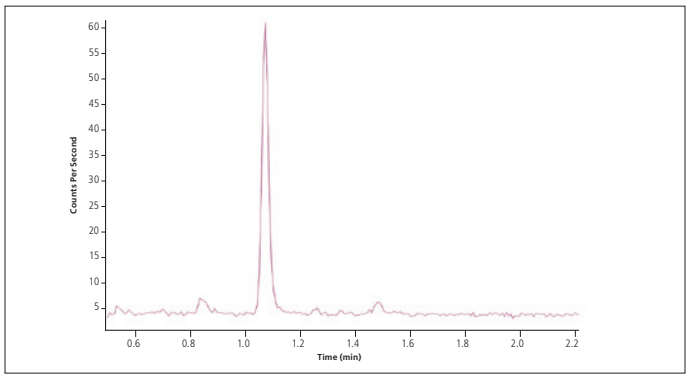


*Figure 4.* Standard injection of 25-hydroxyvitamin $D_3$-$d_6$ at 3 ng/mL.

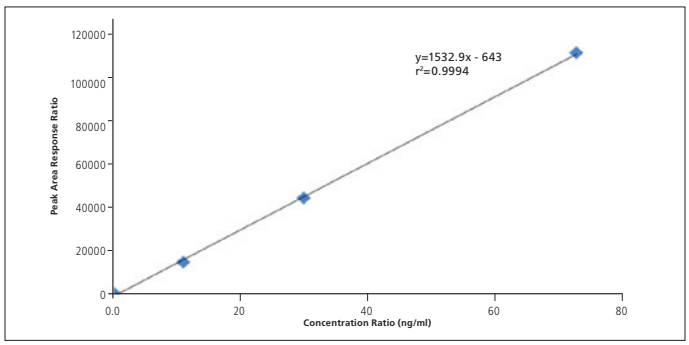


*Figure 5.* Calibration curve for 25-hydroxyvitamin $D_3$ concentration range from blank to 73 ng/mL.

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