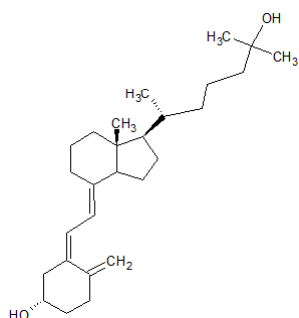


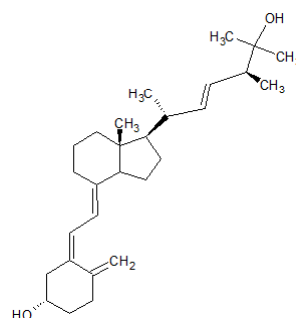
Vitamin D2 and D3 in Serum/Plasma

Vitamin D is a group of fat-soluble secosteroids. In humans, the most important compounds are vitamin D3 (cholecalciferol) and vitamin D2 (ergocalciferol). These compounds can be ingested from the diet, from supplements but also through sun exposure of the skin. Vitamin D is not an essential dietary vitamin since a substance is only classified as an essential vitamin when it cannot be synthesized in sufficient quantities by an organism, and must be obtained from diet.

In the liver, vitamin D3 is converted to 25-hydroxyvitamin D3, whereas vitamin D2 is converted to 25-hydroxyvitamin D2. These are the two specific vitamin D metabolites that are measured in serum and plasma to determine a person's vitamin D status. Analysis of vitamin D's (25-OH D2 and D3) have emerged as a very important clinical method and currently several different patient conditions can be linked to low concentrations of vitamin D's.



25-Hydroxyvitamin D3



25-Hydroxyvitamin D2

We present here a method for analysis of aforementioned molecules using reversed phase LC-MS/MS. Serum and plasma samples from patients were analysed along with standards and control samples. Samples were prepared via simple protein precipitation using acetonitrile containing 1% formic acid and the two internal standards 2H3-25-OH vitamin D2/D3. It is not possible to completely eliminate the endogenous levels of 25-OH Vitamin D2/D3 in human serum/plasma wherefore deuteriated 25-OH Vitamin D2/D3 are needed. These deuteriated internal standards each have six hydrogen atoms replaced with deuterium; 2H6-25-OH vitamin D2/D3. Standard curves are created from standard samples where the ratio of the deuteriated 25-OH vitamin D2/D3 (2H6-25-OH vitamin D2/D3) and the two internal standards 2H3-25-OH vitamin D2/D3 plotted against the ratio of the concentrations of the same.

Vitamin D2 and D3 in Serum/Plasma

Purospher® STAR RP-18 endcapped

Recommended column:

Purospher® STAR RP-18 endcapped (2 µm) Hibar® HR 100-2.1 mm (1.50648.0001)

Recommended solvents and reagents:

Methanol: hypergrade for LC-MS LiChrosolv® (1.06035)

Water: Water for chromatography LiChrosolv® (1.15333)
or freshly purified water from Milli-Q® water purification system

Formic acid 98-100% for analysis EMSURE® ACS, Reag. Ph Eur (1.00264)

Mobile phase: A: Milli-Q water
B: Methanol

| Time (min) | A (%) | B (%) | Flow Rate (mL/min) |
|------------|-------|-------|--------------------|
| 0.00 | 15 | 85 | 0.40 |
| 4.50 | 15 | 85 | 0.40 |
| 4.51 | 0 | 100 | 0.50 |
| 5.53 | 0 | 100 | 0.50 |
| 5.55 | 15 | 85 | 0.40 |
| 6.50 | 15 | 85 | 0.40 |

Quantitation of Vitamin 25-hydroxy-D2 and D3 in plasma/serum

Linear range: 11 – 370 nM (nmol/L).

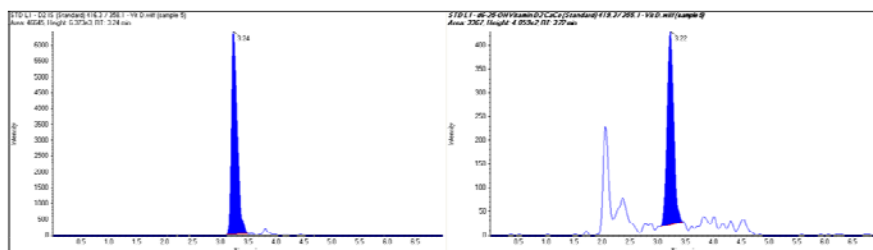
Vitamin D2 and D3 in Serum/Plasma

Purospher® STAR RP-18endcapped

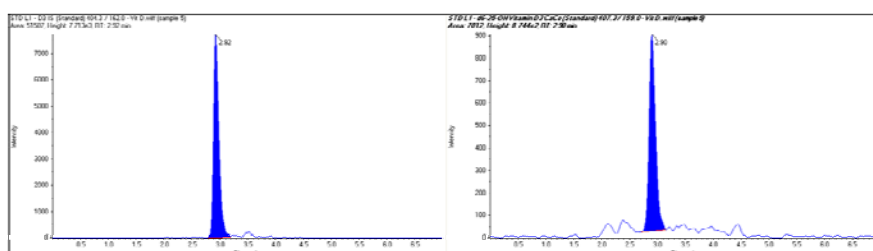
Chromatographic Conditions

| | | |
|---------------|--|----------------|
| Column: | Purospher® STAR RP-18 endcapped (2 µm) Hibar® HR 100-2.1 mm | (1.50648.0001) |
| Injection: | 10 µL | |
| Detection: | LC-MS/MS APCI; MRM transitions: m/z 419.3/355.1, 416.3/358.1 (vitamin D2) and m/z 407.3/159.0 and 404.3/162.0 (vitamin D3) | |
| Flow Rate: | See table | |
| Gradient | See table | |
| Mobile Phase: | A: Milli-Q water B: Methanol | |
| Temperature: | 50 °C | |
| Sample: | Patient urine samples. | |
| Backpressure: | 360 bar (5184 psi) at start of gradient | |

Lowest standard for 25-hydroxyvitamin D2, 12.9 nmol/L, (right chromatogram)
Internal standard D3-25-hydroxyvitamin D2, 50 nmol/L, (left chromatogram)



Lowest standard for 25-hydroxyvitamin D3, 11.3 nmol/L, (right chromatogram)
Internal standard D3-25-hydroxyvitamin D3, 50 nmol/L, (left chromatogram)

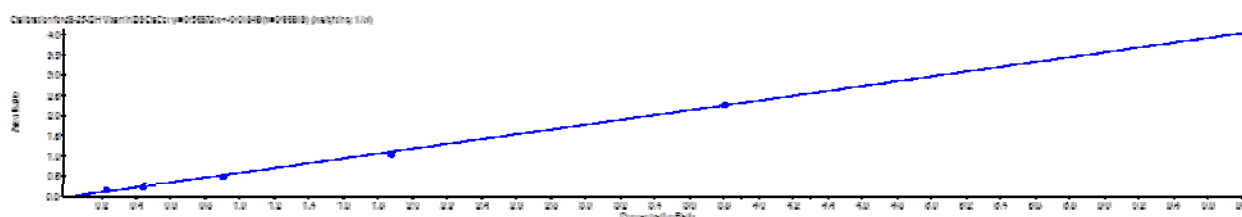
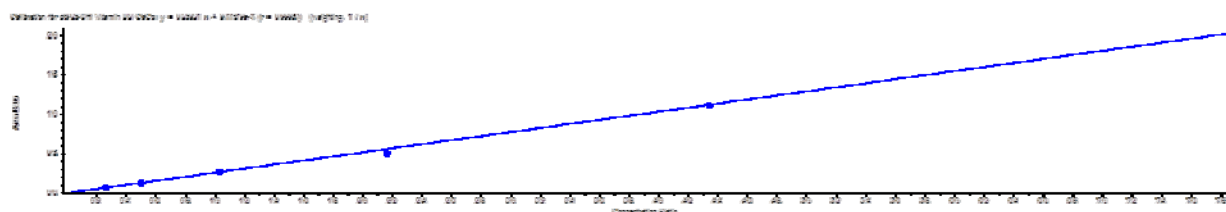


Chromatographic Data

| No. | Compound | Retention Time (min) | Precursor ion (m/z) | Product ions (m/z) |
|-----|------------------|----------------------|---------------------|--------------------|
| 1 | Void volume | 0.5 | – | |
| 2 | 25-OH vitamin D3 | 2.9 | 404.3 | 162.0 |
| 3 | 25-OH vitamin D2 | 3.2 | 416.3 | 358.1 |

Vitamin D2 and D3 in Serum/Plasma

Purospher® STAR RP-18endcapped



Patient samples

