Vitamin D diagnostics by HPLC and LC-MS/MS

It has been known for a number of years that vitamin D’s role in the human organism goes beyond bone metabolism. Determination of vitamin D level is becoming increasingly important from a medical point of view [1-3]. Recent studies have shown that, alongside the well-known functions such as regulation of calcium and phosphate homeostasis, vitamin D also affects the immune system, cell differentiation and numerous other bodily functions [4-6]. The discovery of these other important vital vitamin D functions has increasingly moved its measurement into the focus of routine diagnostics.

In addition, epidemiological studies conducted during the past 25 years have shown a vast extent of low vitamin D levels among the population of different countries, including China, India, and Malaysia [7, 8]. As a result, the parameter so rarely investigated in the past has developed into a “hot” diagnostic marker with millions of tests now, performed worldwide. Hence, monitoring vitamin D status is very important to be able to supplement in response to deficiency states [9-11].

Vitamin D₃ and D₂ – diagnostic relevance

In eukaryotes (with the exception of plants and fungi), vitamin D is synthesised from cholesterol as vitamin D₃. Vitamin D₂, which derives from ergosterol skeleton and occurs in fungi and plants only, can be accessed by humans via food supplements. The industrial production of vitamin D₂ is less of an effort and is common in
North America particularly [12]. Various research studies comparing the receptor activity of vitamin D$_3$ and D$_2$ indicate a significantly reduced metabolic effect of the vitamin D$_3$-VDR complex versus the D$_2$ receptor complex. Although the two vitamin D-VDR complexes seem to bind to the sequences at a similar rate, the vitamin D$_3$ complex is unstable and degrades more rapidly, and is ultimately less effective. Further studies demonstrate that vitamin D$_2$ itself breaks down more rapidly than vitamin D$_3$. Hence, the form of vitamin D that is administered seems to be of major physiological importance. To quantify vitamin D status in routine diagnostic procedures, it must be possible to discriminate at least between these two variants of vitamin D.

**Vitamin D$_3$ and D$_2$ determination**

To establish vitamin D status, it is important to measure both vitamin D$_3$, which is mainly produced in the human body from endogenous precursor molecules induced by UVB radiation, and vitamin D$_2$, which is delivered in the diet (e.g. in mushrooms) in very small amounts. Since the two forms differ in terms of their biological activity (see above), they should be analysed separately and not as a cumulative parameter [1]. The active forms of vitamin D (1,25(OH)$_2$D$_3$ and D$_2$) are unsuitable as markers for determining vitamin D status because of their short half-life of several hours [13]. For this purpose the diagnostic marker of choice is the concentration of 25-OH metabolites (25-OHD$_3$ and 25-OHD$_2$) which are produced in the liver [14]. These metabolites have a longer half-life (several weeks) and are the storage form of D vitamins.

A number of methods are available to determine vitamin D status by measuring concentrations of 25-OHD$_3$ and 25-OHD$_2$. Chromatography and mass spectrometry are the established gold standards [15]. However, even these techniques have been associated in the past with significant variability of results depending on the methods and instruments used. Two of the most essential criteria for reliable testing are:

1. Calibration must be traceable to certified material.
2. The measurement technique must be standardised.

Chromsystems GmbH supplies reagent kits for the determination of 25-OHD$_3$ and 25 OHD$_2$, which address these issues. Additionally, all below-mentioned kits are traceable to the current NIST SRM 972 reference material. This guarantees the accurate quantification of 25-OHD$_3$ and 25 OH D$_2$ concentration.

1. 25-OH-Vitamin D$_3$/D$_2$ in Serum/Plasma (for HPLC)
2. 25-OH-Vitamin D$_3$/D$_2$ in Serum/Plasma ONLINE METHOD (for HPLC)
3. MassChrom® 25-OH-Vitamin D$_3$/D$_2$ in Serum/Plasma (for LC-MS/MS)

The first kit is based on chromatographic separation with UV detection following effective protein precipitation and selective solid phase extraction (Fig. 1). Manual sample preparation and HPLC run times of 12 min are the speed limiting factors.

![Chromatogram of a calibration standard applying the 25-OH-vitamin D$_3$/D$_2$ reagent kit (HPLC).](image1)

The second kit for HPLC significantly reduces time consuming laboratory steps and, thus permits up to 100 analyses per day. Requiring only a small sample volume, this analysis is also suitable for measuring paediatric samples (Fig. 2).

![Chromatogram of a calibration standard applying the 25-OH-vitamin D$_3$/D$_2$ reagent kit (HPLC).](image2)
The “MassChrom® 25-OH Vitamin D₃/D₂ in Serum/Plasma” reagent kit is specially developed to meet requirements of high sample throughput. This kit easily processes, analyses, and produces results on 200-300 samples per day. The high throughput is achieved with a combination of minimised sample preparation and very short analysis times (Fig. 3).

Unlike other analytical approaches, the Chromsystems LC-MS/MS method does not involve time- and labour-intensive liquid-liquid extraction or manual solid phase extraction. Other major benefits include avoidance of neurotoxic solvents such as hexane and a significantly faster sample preparation. In contrast to immunological methods, mass spectrometry allows both analytes – 25-OHD₃ and 25-OHD₂ – to be quantified in the same run with no mutual interference (cross reaction).

Variability is significantly reduced following the introduction of traceable calibration material by Chromsystems. Also in combination with 3PLUS1® Multilevel Calibrators or even 6PLUS1® Multilevel Calibrators, the “MassChrom® 25-OH Vitamin D₃/D₂ in Serum/Plasma” reagent kit gives accurate, precise, reliable, and reproducible results irrespective of which LC-MS/MS system is used. Thus, standardised vitamin D testing contributes to the safe detection of the vitamin D level in the body.

Our portfolio of products includes the possibility of C3-epimer determination for adults as well as for children, especially in infants under the age of one year. Two isotope-labelled internal standards, a 3PLUS1® Multilevel Calibrator Set, and MassCheck® Controls are also available.

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**References**