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Review

# Quantification of circulating 25-hydroxyvitamin D by liquid chromatography–tandem mass spectrometry☆

Michael Vogeser*

*Institute of Clinical Chemistry, Hospital of the University of Munich, Marchioninistr. 15, D-81377 Munich, Germany*

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ABSTRACT

Hypovitaminosis D is a highly prevalent condition and quantification of serum 25-hydroxyvitamin D3 is accepted to be the most useful marker for the assessment of the individual vitamin D status. Due to the increasing awareness of the prevalence and potential health consequences of hypovitaminosis D, the request numbers for 25-hydroxyvitamin D quantification are growing rapidly in many countries. Automated protein binding assays (based on the use of vitamin D-binding protein or antibodies) for the quantification of 25-hydroxyvitamin D3 are available which enable convenient high-throughput analyses in a routine setting; there is, however, substantial concern about accuracy and analytical reliability of these assays. Several LC–MS/MS methods for the quantification of 25-hydroxyvitamin D3 in serum have been described and in a growing number of clinical laboratories this technology is used routinely for vitamin D monitoring. It is justified to assume that LC–MS/MS enables more reliable analyses of 25-hydroxyvitamin D concentrations compared to protein binding assays. In particular the ability to co-quantify the naturally occurring 25-hydroxyvitamin D3 and 25-hydroxyvitamin D2 which is derived primarily from food fortification is a relevant advantage of LC–MS/MS over protein binding assays. This review describes the background of 25-hydroxyvitamin D measurement, compares published LC–MS/MS methods, discusses problems, strengths and limitations of these assays and compares the application characteristics of LC–MS/MS with those of protein binding assays and HPLC-UV.



**Contents**



## 1. Background of 25-hydroxyvitamin D3 measurement

The vitamin D endocrine system plays an essential role in the calcium homeostasis of the body [1,2]. Vitamin D3 (cholecalciferol) is formed from its precursor 7-dehydrocholesterol in the skin by ultraviolet irradiation. In the liver vitamin D3 undergoes hydroxylation to 25-hydroxyvitamin D3, which is further metabolized to the active metabolite 1,25-dihydroxyvitamin D3 in the kidney. Vitamin D3 can also be absorbed from the diet, which is important in case of insufficient sun exposure. Fatty fish naturally contains high amounts of vitamin D3, whereas other foods contain relevant amounts of vitamin D only after fortification. For fortification in many countries vitamin D2 (ergocalciferol) is used, which is derived from plants sources.

Severe deficiency of vitamin D during childhood can cause rickets, a disorder that became prevalent during expansive industrialization and urban migration to "sunless" and polluted cities. During the first half of the 20th century the vitamin D endocrine system was discovered and disturbances of this system were recognized to cause rickets [3]. Eradication of rickets by vitamin D supplementation to children was an essential achievement of modern medicine.

During the following decades, vitamin D deficiency and the vitamin D endocrine system in general was perceived to be predom-

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* Tel.: +49 89 7095 3221; fax: +49 89 7095 6220.
*E-mail address:* Michael.Vogeser@med.uni-muenchen.de.

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inantly linked with the health of the skeletal system in humans. The vitamin D system controls the absorption of calcium from the diet, but only severe hypovitaminosis D is associated with decreased serum calcium. Already latent hypocalcemia, however, leads to increased blood concentrations of the parathyroid hormone (PTH). Consequently, increased PTH can be indicative of milder normocalcaemic forms of vitamin D deficiency. Since increased PTH concentrations are also found in primary hyperparathyroidisms (albeit associated with hypercalcemia) and since PTH shows a poor sample stability, this analyte is not routinely used to assess the vitamin D status. Nevertheless, hypovitaminosis D can be defined as a condition where supplementation of exogenous vitamin D leads to a decrease of PTH concentrations. Measurement of the active vitamin D metabolite 1,25-dihydroxyvitamin D3 in serum, on the other hand, has been found to be not useful in the assessment of the vitamin D status: paradoxically – probably due to secondary hyperparathyroidism – even increased concentrations may be found in hypovitaminosis D [4]. Moreover the concentrations of 1,25-dihydroxyvitamin D3 are extremely low (ng/L range) and measurement is very demanding. For these reasons, quantification of 25-hydroxyvitamin D3 in serum became accepted to be the most useful marker of the vitamin D status. The analyte is highly stable in serum [5].

Laboratory methods applicable to assess the vitamin D system in the setting of routine clinical chemistry became available with the introduction of ligand binding assays for the quantification of 25-hydroxyvitamin D in the 1970s [6,7]. The first competitive protein binding assay (PBA) employing vitamin D-binding protein for analyte binding was introduced 1971. The use of HPLC with UV detection was described in 1977; and a protein binding assay based on antibodies was introduced in 1985.

The constantly increasing use of these routine tests in clinical medicine disclosed a very high prevalence of hypovitaminosis D [2,8]. This also applies to many sunny countries [9–11], either due to traditional local clothing or due to very intense use of sunscreen in the context of prevention of melanoma [12,13] in combination with a sedentary lifestyle. It is assumed that approximately half of the world's elderly have insufficient vitamin D status [14], in particular during autumn and winter.

During the 1990s, research from various areas suggested a significant role of the vitamin D system for health beyond the bone. Since vitamin D receptors were found in a wide variety of tissues, pleiotropic actions of this endocrine system can be assumed. These findings were in line with a growing number of epidemiological studies, linking hypovitaminosis D with diseases such as diabetes, hypertension, arteriosclerosis, cancer, multiple sclerosis, and others [2,15].

Due to the increasing awareness of potential effects of hypovitaminosis D on health, the number of requested analyses again increased substantially and high-throughput analytical methods became desirable. In 2001 the first automated 25-hydroxyvitamin D test was introduced [7]. At present two automated tests are available implemented on multi-channel-random access analyzers (Roche and DiaSorin) and several companies of in vitro diagnostics will soon follow to introduce automated methods. At present besides automated tests still manual immunoassays with radioactive and non-radioactive labels (ELISA) are in use, as well as HPLC-UV methods.

The rapidly growing awareness of the (more or less global) problem of hypovitaminosis D is certainly an important achievement of the past decade in medical research and was based on convenient analytical methods which provide large epidemiological data sets. It is, however, uncertain if widespread measurement of serum 25-hydroxyvitamin D3 is a useful answer to this issue: hypovitaminosis D is reliably avoided by the administration of 1000–2000 IU of vitamin D per day. If strict protection from sunlight is applied aiming to reduce the risk of skin cancer, such vitamin D supplementation may also be necessary during summer [16,17]. Most reliably vitamin D3 (cholecalciferol) is used for this inexpensive supplementation (<3€ per month), which can probably be recommended to all adults at least during the dark months on the northern hemisphere (with dose adaptation in children and adolescents) [15,18]. Given uncompromised gut absorption, measurement of serum 25-hydroxyvitamin D does not seem useful with such standard does of vitamin D [19]. It must be noted that the safe and efficient substitution of 25 μg cholecalciferol for 1 year causes cost per person in the range of a single measurement of 25-hydroxyvitamin D3. Even in otherwise healthy patients with established osteoporosis, measurement of 25-hydroxyvitamin D is generally not assumed to be useful if the recommended supplementation with 25 μg vitamin D daily is reliably administered. Measurement of serum 25-hydroxyvitamin D3 can be useful in case of severe and etiological uncertain osteopenia and in patients with potentially impaired gut absorption (e.g. cystic fibrosis, chronic inflammatory bowl diseases). Furthermore analyses are useful in patients with increased PTH concentrations to differentiate mild hyperparathyroidism from hypovitaminosis D as the cause of increased PTH. Certainly in the context of epidemiological studies quantification of 25-hydroxyvitamin D is of major scientific and public health importance. Optimum vitamin status is currently assumed for serum 25-hydroxyvitamin D3 concentrations above 30 μg/L[1] [20,21]; concentrations below 10 μg/L are considered as severe hypovitaminosis D. Vitamin D-intoxication with concentrations above 150 ng/mL which can be associated with hypercalcemia is found extremely rarely [2].

Regardless of these above considerations, 25-hydroxyvitamin D screening of healthy individuals was promoted intensively during recent years by many physicians and commercial laboratories mainly in the USA. Consequently, 25-hydroxyvitamin D testing became one of the economically most interesting assays for laboratory test providers.

It was clearly recognized from the 1970s on that quantification of 25-hydroxyvitamin D3 in serum is a particular analytical challenge due to several issues [6,7,22]: the analyte is bound to vitamin D-binding protein with high avidity, and a complete release from this bond is essential for reliable results. This is best achieved by serum precipitation with organic solvents as acetonitrile. In fully automated assays the complete release of 25-hydroxyvitamin D from its bonds is difficult to obtain since organic solvents are incompatible with analytical antibodies. In general, competitive protein binding assays are rather sensitive to matrix effects, much more than applies for double-antibody sandwich immunoassays which can be applied for analytes with a larger number of potential epitopes. Furthermore, differential cross-reactivity of ligand binders to naturally occurring 25-hydroxyvitamin D3 and 25-hydroxyvitamin D2 of pharmaceutical origin was recognized as a potential confounder of 25-hydroxyvitamin D results in regions where vitamin D2 is used [23].

Whereas GC–MS reference methods had been developed for the validation and in the context of standardisation of clinically used routine assays for steroid hormone quantification (e.g. testosterone, cortisol), the implementation of GC–MS reference methods for the standardisation of 25-hydroxyvitamin D was found to be extremely complex and was not widely used in assay development or in quality assurance programs. This sub-optimal standardisation and validation of 25-hydroxyvitamin D measurement by ligand binding assays became more and more perceived from 2000 on when automated assays became available. Among

[1] Conversion of unit: 25-hydroxyvitamin D3: [μg/L] × 2.5 = [nmol/L].