CHAPTER TWO

Vitamin D Testing—Where Are We and What Is on the Horizon?

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Abstract

Vitamin D testing is part of laboratory practice since more than 30 years but has become a routine parameter only recently, due to a highly increasing amount of research in the field resulting in new clinical applications. Vitamin D actually represents a family of molecules of which 25OH Vitamin D and 1,25(OH)2 Vitamin D, under their D3 and D2 forms, are the most important to date. Physical detection methods and immunoassays exist for both molecules and are being reviewed and discussed. New developments in the measurement of C3-epi-25OH Vitamin D, 24,25(OH)2 Vitamin D, and free/bioavailable 25OH Vitamin D are also presented. The future of Vitamin D testing is considered based on the evolution of laboratories and based on the scientific research that is currently performed.

1. INTRODUCTION

Vitamin D is part of the laboratories tests menu since the 1980s. It has been used during three decades in the diagnostic and monitoring of Vitamin D deficiency linked to bone diseases such as rickets, osteoporosis,
and osteomalacia [1–3]. Although bone-related issues remain the main field of interest for the measurement of Vitamin D metabolites, the massive amount of research that has been performed during the last decade has led to a number of novel clinical applications, with a large portion still being to be fully proven by large random clinical trials [4–6]. This continuously increasing interest for the action of Vitamin D and for the implications of its deficit has led over time to a real explosion of the number of tests performed by the laboratories.

Clinicians must understand the fundamental difference between 25OH Vitamin D and 1,25(OH)₂ Vitamin D, which is not always the case as 1,25(OH)₂ Vitamin D is still largely misordered [7]. In addition, several immunoassay types and physical detection methods exist for both parameters, and professionals must understand the performance and the limitations of each technique in order to correctly interpret the results of the analyses, together with all the other clinical and diagnostic tools that they have.

Finally, the physiology and metabolism of Vitamin D is not fully understood yet and novel assays are currently being developed with the objective of closing this gap.

This chapter aims at clarifying the different aspects of Vitamin D testing, from a scientific and from an operational perspective. Existing methods are being reviewed and compared, and research assays are also being discussed.

2. MEASUREMENT OF VITAMIN D METABOLITES

Vitamin D is the parent compound of the Vitamin D family (Fig. 1) [8]. As for other Vitamin D metabolites, they exist under two forms, the D₂ and D₃ forms. Vitamin D₃ is synthesized in the skin from 7-dehydrocholesterol under the action of the sun’s UVB radiation [9]. This pathway represents around 90% of the natural sources of Vitamin D. The remaining 10% come from food such as fat fishes, e.g., salmon, sardines, tuna, and eggs. Nowadays it is also common to find in fortified food such as cow’s milk, tofu, cereals, and orange juice. The third source of Vitamin D is the supplementation that is classically prescribed to Vitamin D deficient individuals. Vitamin D₂, on the other hand, is less ubiquitous and is not produced in the human body. It is found in very low quantities in some mushrooms and is mostly limited to high-dose supplements in some countries (Fig. 2) [10].

Once present in the human body, Vitamin D is rapidly metabolized into 25-hydroxyvitamin D (25OH Vitamin D) in the liver [11]. This
transformation is executed by an enzyme from the cytochrome P family named CYP2R1. 25OH Vitamin D has a long half-life of 2–3 weeks, compared to the other Vitamin D metabolites, and is so far the best biomarker to evaluate the Vitamin D status of an individual [12].
25OH Vitamin D is further metabolized into the biologically active 1,25(OH)\(_2\) Vitamin D through the action of the cytochrome P family enzyme CYP27B1. Historically, the kidney was thought to be the only organ where this oxidation takes place. Recent studies have identified the presence and the role of CYP27B1 in many other tissues and organs and that should explain the autocrine/paracrine actions of Vitamin D on the human body [11].

The metabolic pathway of Vitamin D is summarized later starting from the different Vitamin D sources down to the catabolic pathway that takes place through an hydroxylation reaction on the position 24 of either 25OH Vitamin D or 1,25(OH)\(_2\) Vitamin D (Fig. 3).

Serum is the most important matrix for the measurement of Vitamin D metabolites. Serum has the advantage of not being contaminated with anticoagulants used for plasma collection, such as EDTA, Heparin, or Citrate. Vitamin D assays being typically sensitive to interferences from sample matrices, these substances have the potential to interfere with each individual assay and appropriate validation must be conducted when plasma is considered for testing.

Saliva has been explored by a few research groups using either competitive protein-binding assays [13], immunoassays [14], or physical detection methods [15,16]. Results are not consistent within the different publications and the work of Higashi et al. seems to be the most reliable so far. LC–MS has been used by the Japanese group to quantify 25OH Vitamin D in the saliva of healthy volunteers. They have established a correlation between salivary and serum levels of 25OH Vitamin D, and a postulate is made on the salivary 25OH Vitamin D concentration reflecting the levels of free 25OH Vitamin D in serum.

The excretion of Vitamin D metabolites in urine and bile has been observed in adult rats and might be elevated in chronic renal failure [17]. This can be one of the causes of Vitamin D deficiency in this population.

Methods also exist to quantify Vitamin D metabolites in food and supplement matrices [18]. HPLC and LC–MS are mainly used by the manufacturers of fortified food and Vitamin D3 and D2 supplements to assess the concentration of the nonhydroxylated Vitamin D in their products.

### 2.1 Measurement of 25OH Vitamin D
As stated in Section 1 of this chapter, 25OH Vitamin D is so far the best biomarker to evaluate the Vitamin D status of individuals. The measurement...
Fig. 3  Vitamin D metabolism.
of 25OH Vitamin D is useful in the diagnosis of Vitamin D insufficiency or deficiency, to help identify individuals who may benefit from Vitamin D supplementation to reach optimal levels, in monitoring response to Vitamin D supplements for the treatment of bone-related diseases, such as rickets (children), osteomalacia, postmenopausal osteoporosis, and renal osteodystrophy or nonbone-related diseases, and in the diagnosis of Vitamin D toxicity, e.g., patients with suspected toxicity (hypercalcemia) [19–24].

Published in 1971, the very first assay used the competitive protein-binding principle with the native Vitamin D-binding protein (VDBP or DBP) as the capture protein [25,26]. The first immunoassay was developed later in 1985 and used a polyclonal antibody and radioactive I125 as the label [27]. Since that time, other methods have emerged and are discussed in this section.

Current methods for measuring 25OH Vitamin D are classified into three categories. Physical detection methods, which include high-pressure liquid chromatography (HPLC) and liquid chromatography tandem mass spectrometry (LC–MS/MS), are discussed first. The second subsection examines immunoassay methods, covering radioimmunoassays (RIAs), enzyme-linked immunosorbent assays (ELISAs), chemiluminescent immunoassays (CLIsAs), lateral flow immunoassays, and assays for clinical chemistry analyzers (CCA) (Fig. 4).

**Fig. 4** Methods for measuring 25OH Vitamin D: Methods for measuring 25OH Vitamin D can be classified in physical detection methods and immunoassays.
It should be emphasized that the demand for 25OH Vitamin D tests has truly spiraled over the last decade. Different factors can explain this including the tremendous amount of research that is done on Vitamin D and the discovery of nonbone-related actions of Vitamin D. This is illustrated by the figure later plotting the number of publications on Vitamin D each year, in comparison to other vitamins (Fig. 5).

The volume of writings on Vitamin D has clearly risen for more than 10 years with an acceleration in the last 5 or 6 years.

The next figure further reveals this trend and shows the number of 25OH Vitamin D tests ordered every month at two institutions in the United States over the years (Fig. 6) [28].

Fig. 5 Number of publications Vitamin D: Number of publications for Vitamin A, C, and D, from 2004 to 2015. From Pubmed including only the field title.

Fig. 6 Increasing Vit D tests: Average number of 25(OH)D orders per month (in 6-month bins) at Weill Cornell Medical College (WCMC) and University of Iowa Hospitals and Clinics (UIHC).
As stated by Genzen et al., the increase in 25OH Vitamin D testing was most prominent between 2008 and 2010 with annual growth rates of 52–115% between 2008 and 2009.

2.1.1 Physical Detection Methods—25OH Vitamin D

High-pressure or high-performance liquid chromatography (HPLC) and LC–MS/MS are part of this category. Both methods are based on LC to physically separate the different Vitamin D metabolites and the other molecules present in human serum or plasma. LC uses the difference of affinity of the molecules toward the solid phase, contained in the so-called column, or toward the liquid phase, a mixture of organic solvents and/or water-based solutions, known as eluent. The polarity but also the size and conformation of the molecules play an important role in this process (Fig. 7).

The detection of the separated molecules is different between HPLC and LC–MS. While HPLC uses UV to detect and quantify, i.e., the Vitamin D metabolites [29], LC–MS relies on MS to differentiate between entities and to quantify them [30–32]. UV is a powerful detection method, especially for Vitamin D metabolites, thanks to their strong absorption at 264 nm. The triene moiety present in the molecule is responsible for this high signal and most of the Vitamin D metabolites do incorporate this particular functionality in their structure. However, the various Vitamin D metabolites exhibit similar UV patterns and need to be fully separated by the LC step in order to be detected and quantified separately. In other words,

Fig. 7 HPLC: Principle of liquid chromatography. A sample containing different analytes is loaded onto the solid phase and an eluent is added. The analytes will progress according to their affinity for the solid phase and for the eluent. In this example, the light gray circles analytes have a higher affinity for the solid phase than the triangle light gray analytes.
two Vitamin D molecules which would not be fully resolved by the LC part of the method cannot be independently quantified and only the sum of both metabolites can be reported. This is where MS presents a big advantage. In this technique, molecules are detected by their mass-to-charge \((m/z)\) ratio and most of the Vitamin D metabolites have different masses (Fig. 8). They can therefore be separately detected and quantified even if the LC part of the method does not fully resolve them. For molecules having exactly the same mass and having similar affinities for the chromatography solid phase, fragmentation patterns that occur in advanced LC–MS systems are usually different for each individual compound and allow for separated detection and quantification (Fig. 9). This is the case for 1,25(OH)₂ Vitamin D and 24,25(OH)₂ Vitamin D, for example.

HPLC, and LC–MS even more so, are powerful methods for the quantification of 25OH Vitamin D in human fluids. Benefits of using these techniques for the measurement of 25OH Vitamin D are their high sensitivity (typically below 1 ng/mL for LC–MS), high accuracy, and excellent reproducibility profile (CVs typically range from 2% to 7–8%). Thanks to the detection by MC, LC–MS allows for a good resolution of the multiple Vitamin D metabolites, which makes the method less prone to specific sample interferences such as the one caused by the presence of 24,25(OH)₂

<table>
<thead>
<tr>
<th>Metabolites</th>
<th>Monoisotopic mass</th>
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<tbody>
<tr>
<td>Vitamin D3</td>
<td>384.33</td>
</tr>
<tr>
<td>Vitamin D2</td>
<td>396.33</td>
</tr>
<tr>
<td>25OH Vitamin D3</td>
<td>400.33</td>
</tr>
<tr>
<td>25OH Vitamin D2</td>
<td>412.33</td>
</tr>
<tr>
<td>3-epi-25OH Vitamin D3</td>
<td>400.33</td>
</tr>
<tr>
<td>3-epi-25OH Vitamin D2</td>
<td>412.33</td>
</tr>
<tr>
<td>1OH Vitamin D3</td>
<td>400.33</td>
</tr>
<tr>
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<td>24,25(OH)₂ Vitamin D3</td>
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<td>24,25(OH)₂ Vitamin D2</td>
<td>428.33</td>
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<tr>
<td>25,26(OH)₂ Vitamin D3</td>
<td>416.33</td>
</tr>
<tr>
<td>25,26(OH)₂ Vitamin D2</td>
<td>428.33</td>
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</tbody>
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Fig. 8 Vitamin D MW: Monoisotopic masses of several Vitamin D metabolites.
Vitamin D. HPLC and LC–MS are also able to separately quantify the D2 and D3 forms of 25OH Vitamin D, which represent an interest for specific clinical studies and monitoring of patients supplemented with Vitamin D2. Thanks to its excellent performances LC–MS is currently recognized as the Gold Standard methodology for the measurement of 25OH Vitamin D in human fluids such as serum and plasma. The Vitamin D Standardization Program (VDSP) recognizes three Reference Measurement Procedures (RMPs) (Ghent, NIST, and CDC), which are all LC–MS methods [33,34]. This program aims at promoting the standardization of all 25OH Vitamin D assays, one part being assays produced by manufacturers, and the other part being methods developed by laboratories. The Vitamin D Standardization Certification Program (VDSCP), coordinated by the Centers for Disease Control and Prevention (CDC), is part of these efforts and offers to assay manufacturers and laboratories the possibility to certify the mean accuracy and reproducibility of their methods based on the measurement of multiple sets of blinded samples assessed by one of the Reference Measurement Procedures [35].

However, HPLC and LC–MS also present some drawbacks, which are mainly of a technical nature. They both require the access to high quality water, solvents, and chemicals, and most of the laboratories are developing and maintaining their in-house methods, which poses regulatory challenges.

**Fig. 9** HPLC–LC–MS: Example of a chromatogram of two analytes that are not resolved by liquid chromatography. UV cannot differentiate the two molecules, while MS can discriminate them based on their mass-to-charge ratio.
to the concerned authorities and require time and highly skilled engineers. The cost of an instrument and its maintenance are also often a hurdle in several parts of the world. Last but not least, HPLC and LC–MS are sensitive instruments and serum or plasma needs to be extracted, or at least cleaned up, before analysis. This represents a cumbersome and labor intensive process and should not be neglected when considering the use of these methods for the analysis of hundreds or thousands of samples a day.

In terms of performance, C3-epi-25OH Vitamin D remains an issue for many HPLC and LC–MS methods [36]. Because of its similar UV pattern and identical monoisotopic mass and fragmentation patterns it cannot be resolved from 25OH Vitamin D by the UV or MS detection techniques. Therefore, the development of a LC protocol that fully separates the C3-epi metabolite from the desired 25OH Vitamin D is required. Although technical solutions exist and are used by several laboratories, C3-epi-25OH Vitamin D still interferes in many HPLC and LC–MS methods [37]. This was demonstrated in the NIST/NIH Vitamin D Metabolites Quality Assurance Program shown later (Fig. 10). The blinded study sample SRM 972a level 4 was fortified with 3-epi-25-hydroxyvitamin D3 and participants were asked to provide the total concentration of 25OH Vitamin D. LC methods that do not chromatographically separate the 3-epi-25(OH)D3 yield biased results. The majority of the IA methods, on the other hand, do not have cross-reactivity with the 3-epi-25(OH)D3 metabolite and yield an unbiased median result.

The technique is evolving quickly and recent developments have been directed toward even more specific methods, multiplexing, and automation of the laborious extraction steps. Jones et al. have developed a LC–MS method that uses derivatization to increase sensitivity and allows the complete profiling of six Vitamin D metabolites using less than 100 μL of sample [38]. The method can be used in human clinical research as well as in animal basic research. Geib et al. reported the use of commercially available 96-well extraction plates for the “one-pot” preparation of serum samples prior to LC–MS analysis [39]. Although the method does not allow for a fully automated sample treatment coupled to LC–MS, it represents one of the multiple efforts to simplify and robotize the cumbersome sample pretreatment steps associated with physical detection methods.

2.1.2 Immunoassays—25OH Vitamin D

Immunoassays, i.e., assays based on antibodies, for the measurement of 25OH Vitamin D exist since 1985 and are still the most prevalent methods
Fig. 10 Figure from NIST: From NIST/NIH Vitamin D Metabolites Quality Assurance Program Report of Participant Results: Summer 2013 Comparability Study (Exercise 7): The blinded study samples consisted of two vials, Vial A and Vial B. Vial B was SRM 972a Vitamin D metabolites in human serum level 4 (SRM 972a L4), which contains endogenous levels of 25-hydroxyvitamin D2 (25(OH)D2) and 25-hydroxyvitamin D3 (25(OH)D3), but was fortified with 3-epi-25-hydroxyvitamin D3 (3-epi-25(OH)D3). Participants were asked to provide individual concentration values for 25(OH)D2, 25(OH)D3, and 3-epi-25(OH)D3 along with a total concentration of 25(OH)D (25(OH)D total = 25(OH)D2 + 25(OH)D3) for the control and each study sample. The results from immunoassay methods are displayed with open dark gray circles (left of the graph), and the results from the LC-based methods are displayed with open light gray circles (right of the graph). For both of the major techniques (IA or LC), the solid lines represent the consensus median, and the dashed lines represent the approximate 95% confidence interval. The red lines represent the NIST value and its associated uncertainty (i.e., value ± U95). The LC results are bimodal, where nine reported results agree well with both the NIST value and the reported IA results, but the majority of the LC results (73%) are biased high. SRM 972a L4 was fortified with 3-epi-25(OH)D3, and the NIST-certified value for this vitamin D metabolite is 26.4 ± 2.1 ng/mL. The biological significance of 3-epi-25(OH)D3 is uncertain, and this metabolite is not included in the 25(OH)D total concentration. Therefore, LC methods that do not chromatographically separate the 3-epi-25(OH)D3 yield biased results for 25(OH)D3, and hence 25(OH)D total because the 3-epi-25(OH)D3 and the 25(OH)D3 are diastereomers that are detected by the same multiple reaction monitoring (MRM) ions in MS/MS and absorbance wavelength in UV. The majority of the IA methods, on the other hand, do not have cross-reactivity with the 3-epi-25(OH)D3 metabolite and yield an unbiased median result of 29.6 ng/mL for 25(OH)D total in SRM 972a L4.
nowadays, although physical detection methods and more particularly LC–MS are more and more present in clinical laboratories. Immunoassays can be classified into three big categories: RIA, ELISA, and CLIA. All three methodologies are competitive immunoassays, i.e., the 25OH Vitamin D present in the sample competes with a labeled 25OH Vitamin D analogue for a limited number of binding sites on an antibody. They differ in the labeling of the competitive antigen and in the detection method. RIA is based on radioactive iodine 125 labeled 25OH Vitamin D analogues and relies on gamma counters for the detection part. ELISA is based on 25OH Vitamin D analogues labeled with an enzyme. Horseradish peroxidase (HRP) is mainly used followed by alkaline phosphatase (ALP). The detection is based on a colorimetric reaction and is quantified by measuring the absorbance in an ELISA reader. CLIA methods are also based on 25OH Vitamin D analogues labeled with an enzyme but the detection is based on the emission of light by a specific substrate and is quantified by a photometer.

25OH Vitamin D immunoassays are made up of two main technological components (Fig. 11). The first one is the release of 25OH Vitamin D from its binding proteins, namely the VDBP or DBP and albumin. The second part is the complexation of the 25OH Vitamin D by the antibody and its competition with the labeled 25OH Vitamin D analogue. Both parts will be discussed later.

25OH Vitamin D is hydrophobic and poorly soluble in human blood. It is therefore linked to two binding proteins, the VDBP or DBP and the albumin. It is necessary to release 25OH Vitamin D from these two proteins prior to analysis and multiple methods have been developed for this purpose (Fig. 12). Historically organic solvents were used to denature and precipitate

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Fig. 11 25OH Vitamin D immunoassays: Principle of 25OH Vitamin D immunoassays. Left: Release of 25OH Vitamin D from its binding proteins. Right: Complexation of the 25OH Vitamin D by the antibody and its competition with the labeled 25OH Vitamin D analogue.
the serum or plasma proteins, including DBP and albumin [27]. After a separation step 25OH Vitamin D and other hydrophobic molecules were present in the solvent part and could be analyzed with or without additional manipulation steps (Fig. 12A). This method is very efficient and reliable but not compatible with large automated instruments and high-throughput methods. It has been slowly abandoned and other methodologies have been developed over the last 10 years to meet the needs for automated and faster assays. Digestion enzymes such as Proteinase K have been used to decompose the binding proteins and release 25OH Vitamin D in the digestion buffer (Fig. 12B) [40]. This method has not been extensively used because of relatively slow kinetics, the need for optimum enzyme temperature (around 37°C) and the requirement to neutralize the activity of the enzyme in order to avoid the digestion of the antibody itself. The modification of the protein structure through a pH shift is one of the most widely used methods nowadays [41–44]. Modification of the sample pH to mildly acidic or alkaline pH causes an unfolding of the protein structure, releasing the 25OH Vitamin D molecule (Fig. 12C). The pH is then restored to an optimal pH to match the antibody activity. The neutralization of acidic or alkaline pH usually requires an additional step although ingenious methods have been developed to realize this in situ. There is a risk that proteins refold once the pH has been restored and that 25OH Vitamin D is captured by the
refolded protein and not by the antibody. For these reasons, displacement reagents have been developed very recently [45,46]. These reagents act by displacing the 25OH Vitamin D from its binding site at neutral pH and without any major modification of the protein structure. In presence of the displacement reagent, the binding protein cannot link the 25OH Vitamin D anymore and the immunoreaction with the antibody can take place smoothly (Fig. 12D).

Enzymatic digestion of the sample binding proteins was described by Armbruster et al. in 2008 [40]. The serine protease Proteinase K performs the digestion of the sample at 37°C and sodium dodecyl sulfate (SDS) or 8-ANS (8-anilinonaphthalene-1-sulfonic acid) is added to ensure a complete release of 25OH Vitamin D from VDBP and albumin. The activity of the enzyme is then stopped by dilution in a specific buffer containing a calcium scavenger.

To illustrate the use of pH shift without the need for a neutralization step, the application filed by Antoni and Vogl describes an ingenious method [47]. The sample binding proteins are denatured by the use of mildly to highly alkaline conditions, releasing the 25OH Vitamin D. At the same time ethylene carbonate is decomposed by the high-pH solution and acidic HCO₃⁻ ions are formed. The pH is consequently restored to values around 8.5–9.5 within 10 min.

Acidic pH shift has been developed by Yuan et al. in their 25OH Vitamin D assay for CCA [48]. A solution of sodium acetate decreases the pH down to 3.0, releasing the 25OH Vitamin D from its binding proteins. A neutral pH is then restored thanks to the addition of a sodium phosphate buffer.

In its application, Uchida et al. described the use of a buffer solution containing a surfactant that has a steroid skeleton combined with another denaturing agent [49]. The best results were obtained when using a mixture of SDS and either CHAPS (3-(3-cholamidopropyl)dimethylammonio-1-propanesulphonate) or DC Na (deoxycholate sodium). An efficient release was also obtained when mixing the sample with a combination of guanidine hydrochloride and DC Na.

After 25OH Vitamin D has been released from its binding proteins different biological molecules can be involved in immunoassays, including polyclonal antibodies, monoclonal antibodies, and VDBP.

Polyclonal antibodies have been used in 25OH Vitamin D immunoassays for more than 30 years. They are produced by immunization of animals with a 25OH Vitamin D analogue coupled to a carrier protein, then
recurrent bleedings of these animals and isolation of the serum containing the antibodies against 25OH Vitamin D, also called antiserum [27,50]. Rabbits and goats are the most common animals but chickens, guinea pigs, hamsters, horses, mice, rats, and sheep can also be used. Multiple animals are typically involved as the immune system of each animal is different and will lead to polyclonal antibodies having different properties. Before proceeding with the bleedings small serum samples are collected from each animal to evaluate the level of 25OH Vitamin D antibodies present and their performance profile. The specificity of the antibodies, i.e., the cross-reactivity against various Vitamin D metabolites, is one of the key criteria during the animal selection process. Modern 25OH Vitamin D assays must be able to quantify 25OH Vitamin D3 and D2, as both molecules can be present in patient samples, without interference from 1,25(OH)2 Vitamin D; 24,25(OH)2 Vitamin D; 25,26(OH)2 Vitamin D; and C3-epi-25OH Vitamin D. Polyclonal antibodies typically lack the requested specificity, although some examples of specific polyclonal antibodies have been reported. The major drawback of polyclonal antibodies is the impossibility to reproduce them identically. Large volumes of antiserum can be generated from one animal or even from pooling different animal bleedings with similar performance profiles. However, the increasing demand for 25OH Vitamin D test has required assay manufacturers to scale-up their production processes and larger and larger volumes of antibodies are used nowadays, making the use of polyclonal antibodies a hazardous situation for companies willing to supply 25OH Vitamin D assays with stable quality over time.

Monoclonal antibodies relieve this issue [51]. The first step of their development is identical to the polyclonal antibodies although mice are used in most cases. Animals are immunized with a 25OH Vitamin D—protein conjugate, then screened on a regular basis by evaluation of serum samples. Once the required response is obtained, the animals are sacrificed and the spleen cells are collected and fused with myeloma cells to form a hybridoma. The hybridoma is immortal and can be stored in liquid nitrogen for life. A screening process ensures that the selected hybridoma feature the requested performance characteristics. When needed an aliquot of the hybridoma is cultured with classical cell culture techniques and monoclonal antibodies can be harvested and purified within a few weeks. Thanks to this technique, highly specific monoclonal antibodies can be produced on demand and with a perfect lot-to-lot consistency. Most of the contemporary immunoassays use monoclonal antibodies or VDBP as described later.
The third category of biological molecule that can be used in 25OH Vitamin D immunoassays is the binding proteins [52]. Extracted native VDBP or its recombinant form both have the ability to capture the released 25OH Vitamin D from the samples. The role of VDBP in the human body is to transport and store multiple Vitamin D metabolites. It has therefore the capacity to bind both the D2 and the D3 forms of 25OH Vitamin D. However, interferences from other Vitamin D metabolites can be foreseen although the affinity toward the two forms of 25OH Vitamin D is the highest.

Depending on the assay format the antibodies or the binding proteins are immobilized on a plastic surface or on paramagnetic particles.

Released 25OH Vitamin D from the patient samples will compete for the biological molecule with a labeled 25OH Vitamin D analogue. RIA use a radioactive isotope of iodine namely iodine 125 as the label. Iodine 125 is incorporated on the 25OH Vitamin D analogue onto a tyramine or a histamine moiety by electrophilic addition. As the iodine 125 is relatively small compared to the 25OH Vitamin D analogue structure the competition with the released 25OH Vitamin D is very well balanced and that is the reason why RIA are still among the most sensitive assays. ELISAs involve HRP or ALP, two enzymes capable of transforming a substrate into a product with the formation of color. As both HRP and ALP are large biological molecules, the balance of the competition between the native and the labeled 25OH Vitamin D is disturbed in favor of the native antigen and assay development tricks must be used to overcome this issue. For this reason, however, ELISAs are usually less sensitive than, e.g., RIA. CLIAs are also based on enzyme conjugates but the substrates are different and produce the emission of light that is read by a photometer.

Other assay formats exist in which the antibodies are present in the liquid phase and are precipitated using a neutral solid phase such as long-chain polyethylene glycol [53]. The Vitamin D analogue can also be immobilized on a solid phase and the antibody is therefore labeled [54]. More recently sandwich-based immunoassays have emerged [55]. A classical sandwich immunoassay is not possible for small molecules such as 25OH Vitamin D, as only one paratope is available for complexation by a single antibody. But the change of conformation of an antibody upon complexation of 25OH Vitamin D can be exploited to be bound by a second antibody. The latter only recognizes the first antibody conformationally altered by the presence of 25OH Vitamin D.
Finally, the position of the 25OH Vitamin D molecule on which the carrier protein for immunization or the label is conjugated is crucial. Immunization of animals with a 25OH Vitamin D analogue conjugated to the hydroxyl group on position 3 will produce antibodies directed toward the opposite side of the molecule, i.e., the side chain. These antibodies will be very selective for different Vitamin D metabolites bearing different side chains but will suffer from a lack of selectivity for Vitamin D metabolites that differ from the position 3 region [27]. On the other hand, antibodies produced from a protein conjugated on the side chain will have an opposite specificity profile [51]. In both cases, the labeled 25OH Vitamin D analogue must be conjugated at the same position as the immunogen used during the antibody development so that the same region of the molecule is exposed to the antibody during the assay.

The figure later shows the distribution between the different methods participating in the Vitamin D External Quality Assessment Scheme (DEQAS) in July 2012 and in July 2015 (Fig. 13) [56]. Chemiluminescence-based assays (CLIA) represent the biggest part of the pie with a 9% increase over the last 3 years. The fact that these methods are fully automated and have high throughput is certainly one of the main reasons for this success. ELISA was the second method in July 2012 but the number of participants has strongly diminished by more than 50%, while LC–MS has become more and more popular during the same period. RIA has continued to diminish and HPLC and other methods have remained stable at relatively low numbers. It should be emphasized that this does not represent the real distribution of 25OH Vitamin D methods in clinical laboratories worldwide, but it shows at least the general trends in the field.

2.2 Measurement of 1,25(OH)2 Vitamin D

Basics of HPLC, LC–MS, and Immunoassays have been discussed in the previous section on the measurement of 25OH Vitamin D and most of them apply for the measurement of 1,25(OH)2 Vitamin D as well.

In some aspects the measurement of 1,25-dihydroxyvitamin D or 1,25(OH)2 Vitamin D is similar to the measurement of 25OH Vitamin D. Some other aspects are very different.

1,25(OH)2 Vitamin D links in the human body to the Vitamin D receptor (VDR) and is therefore the biologically active form of the Vitamin D family [57]. Because of its rapid production and transformation metabolism, and of its tight regulation by hormonal control, the
concentration of 1,25(OH)$_2$ Vitamin D is not a reliable marker of an individual’s Vitamin D status, and the measurement of 25OH Vitamin D plays this role [12]. However, 1,25(OH)$_2$ Vitamin D can be useful as a second-order test in the assessment of Vitamin D status, especially in patients with renal disease [58], in case of investigation of some patients with clinical evidence of Vitamin D deficiency [59], in the differential diagnosis of hypercalcemia [60], in primary hyperparathyroidism [61], and in physiologic hyperparathyroidism secondary to low-calcium or Vitamin D intake, for
patients with granulomatous diseases [62] and malignancies containing non-regulated 1-alpha hydroxylase in the lesion, and in hypoparathyroidism [63].

The concentration of 1,25(OH)\(_2\) Vitamin D in the serum or plasma of healthy individuals is typically 20–50 pg/mL, that being 1000 times less than the concentration of 25OH Vitamin D and to a lower extent than the concentration of Vitamin D, 24,25(OH)\(_2\) Vitamin D, and other Vitamin D metabolites. Measuring 1,25(OH)\(_2\) Vitamin D is like fishing for the right specimen in a shoal of similar fish. Scientists have, therefore, developed specific extraction and separation methods to allow the quantification of 1,25(OH)\(_2\) Vitamin D without interference from other structurally close molecules.

Contrary to 25OH Vitamin D, 1,25(OH)\(_2\) Vitamin D has much less affinity with the VDBP and the extraction methods ensure an efficient and reliable release.

Immunoassays largely dominate the field, with RIA, ELISA, and CLIA methods. More recently, the physical detection method LC–MS has appeared and is slowly expanding, due to strong technical hurdles (Fig. 14).

Fig. 14 Methods for measuring 1,25(OH)\(_2\) Vitamin D: Methods for measuring 1,25(OH)\(_2\) Vitamin D can be classified in physical detection methods and immunoassays.

2.2.1 Physical Detection Methods—1,25(OH)\(_2\) Vitamin D

LC–MS represents this category [64–68]. Despite its high performances LC–MS suffers from the low sensitivity of the technique against 1,25(OH)\(_2\) Vitamin D. Therefore, derivatization is often used to increase the dose response of the signal. Extraction and separation steps are also mandatory to ensure sufficient specificity and accuracy. Immunoaffinity columns are today the most widely used method to separate 1,25(OH)\(_2\) Vitamin D from the other Vitamin D metabolites. Antibodies against 1,25(OH)\(_2\) Vitamin D are immobilized on a solid phase and specifically capture and
release this molecule according to the elution conditions. On-line sample extraction and 96-well plate solid-phase extraction sample preparation have also been described. Overall the extraction, separation, LC, and MS steps make LC–MS a complex and technically challenging method for the measurement of 1,25(OH)$_2$ Vitamin D.

2.2.2 Immunoassays—1,25(OH)$_2$ Vitamin D

Immunoassays are the most widespread method for the measurement of 1,25(OH)$_2$ Vitamin D. They can be classified into three big categories: RIA, ELISA, and CLIA. The RIA and ELISA methods are competitive immunoassays and are all based on extraction and separation protocols to isolate the 1,25(OH)$_2$ Vitamin D from the other Vitamin D metabolites and various matrix components.

Extraction is generally accomplished with the use of an organic solvent or of a mixture of solvents, such as methanol, acetonitrile, cyclohexane, diisopropyl ether, or ethyl acetate [69,70]. The separation of 1,25(OH)$_2$ Vitamin D can then be performed using two apparently similar but very different techniques. Classical chromatography on silica or C$_{18}$OH cartridges is one of these. The extracted sample is applied on the cartridge and the different molecules present in the extract will progress, or elute, through the solid phase at different speeds, mainly depending on their polarity. The use of multiple specific solvents, or eluents, ensures a clean separation of 1,25(OH)$_2$ Vitamin D from the other molecules. The fraction containing 1,25(OH)$_2$ Vitamin D is then used as such, or after a drying—reconstitution step, in the RIA or ELISA (Fig. 15).

The second technique that is used to isolate 1,25(OH)$_2$ Vitamin D is the immunoaffinity separation [71–73]. Specific antibodies against 1,25(OH)$_2$ Vitamin D are immobilized on a solid phase and the extracted sample is applied through this solid phase. Based on the affinity of the antibodies toward 1,25(OH)$_2$ Vitamin D, this molecule is retained in the cartridge while the others flow through quickly. Conditions that will release 1,25(OH)$_2$ Vitamin D from the antibody are then applied to recover the target molecule and this fraction is used as such, or after a drying—reconstitution step, in the RIA or ELISA (Fig. 16).

Although they may look very similar, the chromatography and the immunoaffinity techniques are very different in their principle as the first rely on different elution speeds according to physicochemical parameters, mainly polarity, while the second uses the capacity of an immobilized antibody to selectively bind 1,25(OH)$_2$ Vitamin D. The specificity of the
Fig. 15 Chromatography 1,25: Separation of 1,25(OH)₂ Vitamin D by chromatography. The extracted sample is applied on the cartridge and the different molecules progress according to their affinity or polarity for the solid phase or for the different eluents.
chromatographic separation depends on the quality of the solid phase and on its packing, and on the strict respect of the elution protocol. On the contrary, immunoaffinity separation is a more robust technique with respect to the experimental conditions, but the specificity of the method is highly dependent on the choice of the antibody during assay development. The comprehension of these differences is necessary when using, comparing, or troubleshooting these methods.

Immunoaffinity can also be applied to automated assays [74,75]. In this case, the separation antibody is immobilized on a paramagnetic particle and different buffers are used to perform the capture or the release of 1,25(OH)$_2$ Vitamin D from the particles (Fig. 17). Washing steps involving a magnet to retain the particles in the bottom of the separation cell lead to the separation of 1,25(OH)$_2$ Vitamin D from the other molecules. The solution containing 1,25(OH)$_2$ Vitamin D is then transferred into a second test cell where another paramagnetic particle coated with an antibody against 1,25(OH)$_2$ Vitamin D is used for the immunoassay part.

Recently, a novel CLIA method has been developed and released on the market. This innovative assay relies on the VDR to selectively bind the 1,25(OH)$_2$ Vitamin D. Due to its high instability in assay conditions a more stable fragment of the VDR is used to capture the 1,25(OH)$_2$ Vitamin D. A conformation change of the VDR fragment occurs upon binding of the 1,25(OH)$_2$ Vitamin D and a supported monoclonal antibody selectively recognizes the conformationally changed molecule. After a washing step under magnetic field, a second antibody directed toward another region of the recombinant VDR is added and contains the label that will lead to the chemiluminescent signal. This novel methodology involves no true physical separation step, and therefore allows the assay to be fully automatable.

**Fig. 16** Immunoaffinity 1,25: Separation of 1,25(OH)$_2$ Vitamin D by immunoaffinity. The extracted sample is applied on a solid phase coated with specific antibodies against 1,25(OH)$_2$ Vitamin D. Based on the affinity of the antibodies toward 1,25(OH)$_2$ Vitamin D, this molecule is retained in the cartridge, while the others flow through quickly.
Fig. 17  Particles immunoaffinity 1,25: Separation of 1,25(OH)₂ Vitamin D by immunoaffinity with particles. The sample is incubated with paramagnetic particles coated with a specific antibody against 1,25(OH)₂ Vitamin D (A). 1,25(OH)₂ Vitamin D is captured by the antibody (B). A magnetic field is applied to attract the particles and the remaining solution is washed away (C). The magnetic field is stopped and 1,25(OH)₂ Vitamin D is released from the particles thanks to a specific buffer (D). The supernatant containing the 1,25(OH)₂ Vitamin D is transferred to a second cuvette and incubated with paramagnetic particles coated with a specific antibody against 1,25(OH)₂ Vitamin D and a labeled 1,25(OH)₂ Vitamin D conjugate (E). The competition takes place and the signal is read after another washing cycle (F).
2.3 Free and Bioavailable 25OH Vitamin D

Due to its hydrophobic nature 25OH Vitamin D, and other Vitamin D metabolites, circulate on binding proteins. About 90% of the total circulating 25OH Vitamin D is bound to the so-called VDBP or DBP. VDBP, also known as group-specific component globulin, is a 58 kDa glycosylated alpha-globulin. It binds Vitamin D metabolites with the following relative affinities: 25OHD-23,26-lactone $>$ 25OHD $= 24,25$(OH)$_2$D $= 25,26$(OH)$_2$D $> > 1,25$(OH)$_2$D $> $ Vitamin D [76–78]. The remaining 10% are bound to albumin, the main protein of human blood plasma. Although the affinity of albumin toward 25OH Vitamin D is much lower than the affinity of VDBP, the high concentration of albumin compensates for this difference. A tiny fraction representing 0.04% of the total 25OH Vitamin D concentration circulates as the free form [79]. The sum of the free form and the fraction bound to albumin is called bioavailable 25OH Vitamin D [80]. The sum of all three fractions is called total 25OH Vitamin D although the term “total” often refers to the sum of the D2 and D3 forms of the Vitamin D metabolites (Fig. 18).

The conversion of 25OH Vitamin D into the biologically active 1,25 (OH)$_2$ Vitamin D takes place into the cells and so requires the internalization of 25OH Vitamin D from the extracellular fluid. Different transport mechanisms are likely to be involved and some of them involve the concentration of the free ligand as one of the important parameters. In these cases, the fraction of free 25OH Vitamin D relates to the biological activity of Vitamin D, and therefore may better reflect the physiological action of Vitamin D than the total concentration of 25OH Vitamin D (Fig. 19) [81].

The fraction of free 25OH Vitamin D represents about 0.04% of the total concentration of 25OH Vitamin D. However, this percentage is not constant and varies according to different conditions. Although the level of

\[ \text{DBP} \quad \text{90\%} \]
\[ \text{Albumin} \quad \text{10\%} \]
\[ \text{Free 25OH Vitamin D} \quad \text{0.04\%} \]
\[ \text{Bioavailable 25OH Vitamin D} \]
\[ \text{Total 25OH Vitamin D} \]

Fig. 18 Free and bioavailable Vitamin D: Free, bioavailable, and total 25OH Vitamin D.
albumin tends to be stable among individuals, the concentration of VDBP can fluctuate in several conditions, therefore, influencing the fraction of free 25OH Vitamin D. Pregnancy leads to an increase of the VDBP levels by about 50% while, e.g., liver failure and chronic kidney disease both result in a decrease of the VDBP concentration by also about 50%. In case of elevated binding proteins concentration, the fraction of free 25OH Vitamin D is lower, and vice-versa in the case of low-binding proteins concentration. In these conditions, and in the conditions listed later, the measurement of free 25OH Vitamin D may be a better marker of the Vitamin D activity than the classical measurement of total 25OH Vitamin D (Table 1).

In addition to variable concentrations VDBP also exists as different polymorphic forms (Table 2). The affinity of the different VDBP forms toward 25OH Vitamin D may vary although this is still under debate [76–78]. A polymorphic form with a high affinity for 25OH Vitamin D will decrease the fraction of free 25OH Vitamin D available. Conversely, a low-affinity VDBP form will result in higher levels of free 25OH Vitamin D. The major form of VDBP present in individuals depends on the ethnicity. For example,
Table 1 VDBP Concentration: Vitamin D-Binding Protein Concentration According to Different Clinical Conditions

<table>
<thead>
<tr>
<th>Clinical Condition</th>
<th>Reported DBP Concentration Range (mg/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal women (Bouillon)</td>
<td>333 ± 58</td>
</tr>
<tr>
<td>Normal men and women (Bikle)</td>
<td>405 ± 128</td>
</tr>
<tr>
<td>Normal</td>
<td>436 ± 33</td>
</tr>
<tr>
<td>Pregnancy</td>
<td></td>
</tr>
<tr>
<td>18 ± 4 weeks</td>
<td>613 ± 142</td>
</tr>
<tr>
<td>32 ± 3 weeks</td>
<td>683 ± 82</td>
</tr>
<tr>
<td>35 ± 1 weeks</td>
<td>688 ± 104</td>
</tr>
<tr>
<td>40 ± 1 weeks</td>
<td>616 ± 84</td>
</tr>
<tr>
<td>Use of oral contraceptive</td>
<td>488 ± 90</td>
</tr>
<tr>
<td>Normal weight women</td>
<td>266 ± 104</td>
</tr>
<tr>
<td>Obese women</td>
<td>320 ± 121</td>
</tr>
<tr>
<td>Liver disease</td>
<td>178 ± 92</td>
</tr>
<tr>
<td>Peritoneal dialysis</td>
<td>203 ± 14</td>
</tr>
<tr>
<td>Nephrotic patients</td>
<td>371 ± 46</td>
</tr>
<tr>
<td>Dialysis population</td>
<td>158 (69–217)</td>
</tr>
<tr>
<td>Pediatric renal disease</td>
<td></td>
</tr>
<tr>
<td>CKD stage 2/3</td>
<td>206 (136–287)</td>
</tr>
<tr>
<td>CKD stage 4/5</td>
<td>208 (101–282)</td>
</tr>
<tr>
<td>Dialysis</td>
<td>168 (99–242)</td>
</tr>
</tbody>
</table>

Table 2 VDBP Forms: Affinity Constants of DBP for 25OH Vitamin D, for the Six Different Phenotypes, According to Arnaud and Constans

<table>
<thead>
<tr>
<th>Diplotype</th>
<th>Phenotype</th>
<th>Binding Coefficient</th>
</tr>
</thead>
<tbody>
<tr>
<td>GC/GC</td>
<td>Gc-1S/Gc-1S</td>
<td>6 × 10^8</td>
</tr>
<tr>
<td>GC/TC</td>
<td>Gc-1S/Gc-1F</td>
<td>4.8 × 10^8</td>
</tr>
<tr>
<td>GC/TA</td>
<td>Gc-1S/Gc-2</td>
<td>8.6 × 10^8</td>
</tr>
<tr>
<td>TC/TC</td>
<td>Gc-1F/Gc-1F</td>
<td>3.6 × 10^8</td>
</tr>
<tr>
<td>TC/TA</td>
<td>Gc-1F/Gc-2</td>
<td>7.4 × 10^8</td>
</tr>
<tr>
<td>TA/TA</td>
<td>Gc-2/Gc-2</td>
<td>11.2 × 10^8</td>
</tr>
</tbody>
</table>
among blacks the predominant form is Gc1F, while among whites, the predominant form is Gc1S. If the difference in affinity really exists, the measurement of free 25OH Vitamin D may again be a better marker of the Vitamin D action than the measurement of total 25OH Vitamin D [89–91].

Free 25OH Vitamin D can be either measured or estimated through calculations. Indirect measurement was first demonstrated by the group of Bikle in 1986 [86]. Centrifugal ultrafiltration of serum samples spiked with radioactive compounds led to the evaluation of the percentage of free 25OH Vitamin D. Its concentration was then calculated from the concentration of total 25OH Vitamin D, as obtained by another assay. Although the methodology proved to be quite accurate and made it possible to establish large differences in the percentage of free 25OH Vitamin D between a normal population and a population of patients presenting liver failures, the technique presented several drawbacks. It required large volumes of samples and the manipulation of tritium and C14-based reagents, and consumed a lot of time and precious manpower.

Direct measurement of free 25OH Vitamin D first became available in 2013 through the development of a super sensitive ELISA [79]. The method presents a limit of detection of <3 pg/mL and a measuring range that covers 0.2–35 pg/mL. Repeatability and reproducibility are representative of the ELISA technology and the assay can detect both free 25OH Vitamin D3 and free 25OH Vitamin D2 (Table 3) [92]. Although the assay has been initially validated for use with human samples, mice samples have also been successfully assessed [93]. As from the 1st of January 2015 the assay has become commercially available, which should allow for a broader use by Vitamin D researchers [94].

**Table 3** Free 25OH Vitamin D ELISA: Performances of the Free 25OH Vitamin D ELISA Assay

<table>
<thead>
<tr>
<th>Free 25OH Vitamin D ELISA Assay</th>
<th>0.2–35 pg/mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Range</td>
<td></td>
</tr>
<tr>
<td>Total precision</td>
<td>10.2% at 6.0 pg/mL</td>
</tr>
<tr>
<td></td>
<td>7.6% at 10.9 pg/mL</td>
</tr>
<tr>
<td></td>
<td>5.5% at 24.9 pg/mL</td>
</tr>
<tr>
<td>LoB</td>
<td>1.9 pg/mL</td>
</tr>
<tr>
<td>LoD</td>
<td>2.8 pg/mL</td>
</tr>
<tr>
<td>Cross-reactivity to 25OH Vitamin D2</td>
<td>77%</td>
</tr>
</tbody>
</table>
As for e.g., free testosterone, free 25OH Vitamin D can also be estimated by calculation methods [92,95]. It requires the quantification of total 25OH Vitamin D, albumin, and VDBP and involves mathematical equations that take into account the affinity of both binding proteins (Fig. 20). Although the measurement of total 25OH Vitamin D and albumin is nowadays increasingly accurate, VDBP assays remain of variable qualities. The main reason is the use of monoclonal antibodies in some of these assays that specifically recognize some VDBP polymorphic forms but not all of them. The true concentration of VDBP is therefore often underestimated with these assays. Furthermore, the affinity of VDBP is thought to be different for each VDBP form and an accurate calculation methodology should ideally include the genotyping of this protein and the use of the proper affinity constant.

Because of these uncertainties the direct measurement of free 25OH Vitamin D is thought to be superior to the calculations, as this was postulated and demonstrated by several research groups [92,95].

Bioavailable 25OH Vitamin D, i.e., the sum of the free fraction plus the fraction that is bound to albumin with a moderate affinity, can also be calculated using algorithms that are similar to those for free 25OH Vitamin D [96]. The same issues than for the calculation of free 25OH Vitamin D have to be taken into account for the estimation of the bioavailable fraction of 25OH Vitamin D.

Direct measurement of bioavailable 25OH Vitamin D has been reported but no commercial assay has emerged, which limits the validation and use of the method [89].

2.4 24,25(OH)₂ Vitamin D

24,25(OH)₂ Vitamin D is formed through the hydroxylation of 25OH Vitamin D by the cytochrome P family enzyme CYP24A1. This metabolite has been seen as a pure catabolite of the Vitamin D metabolic pathway for a long time and has attracted little interest from the research community [97,98]. Recently several LC–MS methods have been developed for the measurement of 24,25(OH)₂ Vitamin D alone, or together with other Vitamin
D metabolites such as 25OH Vitamin D, 3-epi-25OH Vitamin D, and 1,25(OH)₂ Vitamin D.

Kaufmann et al. described a novel LC–MS-based method involving derivatization to simultaneously assay multiple vitamin D metabolites, including 25OH Vitamin D and 24,25(OH)₂ Vitamin D using 100 μL of serum with a 5-min run time. The method was used on more than 1000 normal and disease samples involving vitamin D deficiency or hypercalcemia in addition to studies involving knockout mouse models [99].

More recently a high-throughput supportive liquid–liquid extraction and LC–MS/MS method was developed to quantify 10 Vitamin D metabolites in a single run. The methodology was validated by analysis of human serum samples and used in a cohort of 116 healthy subjects [100].

These novel methods have been used to study the importance of this metabolite, alone or expressed as a ratio 24,25(OH)₂ Vitamin D/25OH Vitamin D [101,102]. This ratio is less than 0.09 in Vitamin D insufficient and deficient subjects (serum 25OH Vit D < 20 ng/mL) but is greatly elevated (80–100) in patients with idiopathic infantile hypercalcemia. While PTH to 25OH Vitamin D plots have been commonly used for estimation of Vitamin D sufficiency, plotting the molar ratio of 25OH Vitamin D:24,25(OH)₂ Vitamin D to 25OH Vitamin D, as proposed recently, is of greater strength than that of the PTH to 25OH Vitamin D.

Decreased 25OH Vit D catabolism can also be measured by lower circulating 24,25(OH)₂ Vitamin D concentration and is associated with increased risks of secondary hyperparathyroidism and possibly with death.

2.5 C3-epi-25OH Vitamin D

C3-epi-25OH Vitamin D or so-called C3-epimer is an epimeric form of 25OH Vitamin D, i.e., a stereoisomer differing by a single chiral center. The hydroxyl function on position 3 of the molecule is inverted while the other chiral centers remain unchanged. C3-epi-25OH Vitamin D is formed through a three epimerization pathway that parallels the standard metabolic pathway [103].

C3-epi-25OH Vitamin D is more abundant in infants under the age of one and is of less amplitude in adolescents and adults. However, highly variable concentrations and prevalence have been reported by several groups, which makes it difficult to evaluate its real importance in patient samples (Table 4) [104–109].
For many years it was questioned whether C3-epi-25OH Vitamin D was as important as its analogue 25OH Vitamin D in the biological activity of Vitamin D in the body. Recent reports revealed that until more studies are completed, it is recommended not to include this molecule in the reporting of the 25OH Vitamin D concentration, or alternatively as a separate report for information only.

Most immunoassays are selective toward C3-epi-25OH Vitamin D, which means that the antibody used in the assay does not cross-react, or to a negligible extent, with the epimer (Table 5) [110–117]. The result of these tests, therefore, only accounts for 25OH Vitamin D, and not for its stereoisomer.
As discussed in a previous chapter, HPLC and LC–MS detection techniques cannot differentiate between 25OH Vitamin D and C3-epimer, which requires the development of LC protocols that fully separate the C3-epi metabolite from the desired 25OH Vitamin D. Nowadays many HPLC and LC–MS assays still falsely include C3-epi-25OH Vitamin D in the quantification of 25OH Vitamin D.

### 3. FUTURE DIRECTIONS

The previous chapters have described the methods for measuring 25OH Vitamin D, 1,25(OH)$_2$ Vitamin D, free and bioavailable 25OH Vitamin D, 24,25(OH)$_2$ Vitamin D, and C3-epi-25OH Vitamin D, although the latter should actually be excluded for the desired measurements.

Future developments in the field of Vitamin D testing can be classified into two categories: improvement of existing methods and development of novel assays.

#### 3.1 Improvement of Existing Methods

Over the last few years, a great deal of effort has gone into standardizing 25OH Vitamin D assays across immunoassays manufacturers and across methods developed by laboratories. The National Institutes of Health (NIH) Office of Dietary Supplements (ODS) established the VDSP in November 2010 [33]. From that time RMPs have been established, commutability studies have been conducted to assess the commutability of,

<table>
<thead>
<tr>
<th>Assay</th>
<th>Cross-Reactivity with C3-Epimer (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DIAsource ELISA</td>
<td>0.07</td>
</tr>
<tr>
<td>IDS ELISA</td>
<td>&lt;1</td>
</tr>
<tr>
<td>IDS CLIA</td>
<td>1</td>
</tr>
<tr>
<td>Siemens CLIA</td>
<td>1.1</td>
</tr>
<tr>
<td>Qualigen</td>
<td>7.8</td>
</tr>
<tr>
<td>Euroimmun ELISA</td>
<td>17</td>
</tr>
<tr>
<td>Bio-Rad</td>
<td>59</td>
</tr>
<tr>
<td>Roche CLIA</td>
<td>91</td>
</tr>
</tbody>
</table>

As discussed in a previous chapter, HPLC and LC–MS detection techniques cannot differentiate between 25OH Vitamin D and C3-epimer, which requires the development of LC protocols that fully separate the C3-epi metabolite from the desired 25OH Vitamin D. Nowadays many HPLC and LC–MS assays still falsely include C3-epi-25OH Vitamin D in the quantification of 25OH Vitamin D.
e.g., standardized reference materials (SRMs) and quality control programs samples such as DEQAS and CAP, across various methods. The CDC initiated the VDSCP as part of these efforts [35]. This program assesses the assay performance (bias and imprecision) of the lab or assay manufacturer throughout 1 year. In March 2016, 12 LC–MS methods and 8 immunoassays were certified by this program. Future directions will include new commutability studies and novel acceptance criteria that combine the mean bias and individual sample-to-sample bias against the RMP. Standardization of the measurement of 25OH Vitamin D–related methods and/or the development of specific RMPs are currently being discussed. This includes 1,25(OH)_2 Vitamin D, 24,25(OH)_2 Vitamin D, C3-epi-25OH Vitamin D, VDBP, and free 25OH Vitamin D.

Very little attention has been paid to the cross-reactivity of 1,25(OH)_2 Vitamin D assays for the D2 form of this metabolite. This is very surprising as a number of considerations have been directed toward the cross-reactivity of 25OH Vitamin D methods for the D2 form and this is still an important topic in quality control programs such as DEQAS and CAP. As Vitamin D2 is converted into 25OH Vitamin D2, then into 1,25(OH)_2 Vitamin D2, without cross-over with the D3 forms, the same attention has to be paid to 1,25(OH)_2 Vitamin D assays. As can be seen from the table later, immunoassays are far from being equal in this respect (Table 6) [118–123].

Finally many efforts are currently directed toward the automation of LC–MS 25OH Vitamin D methods. Although LC–MS has been recognized as the Golden Standard method for the measurement of 25OH Vitamin D, the tedious extraction procedure that systematically takes place before injection on the LC system prevents the use of this methodology in laboratories where high-throughput assays are required. Pipetting platforms have been

<table>
<thead>
<tr>
<th>Assay</th>
<th>Cross-Reactivity with 1,25(OH)_2 Vitamin D2 (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DIAsource ELISA</td>
<td>108</td>
</tr>
<tr>
<td>DiaSorin CLIA</td>
<td>105</td>
</tr>
<tr>
<td>DIAsource RIA</td>
<td>92</td>
</tr>
<tr>
<td>IDS CLIA</td>
<td>75</td>
</tr>
<tr>
<td>Immundiagnostik</td>
<td>41</td>
</tr>
<tr>
<td>IDS ELISA</td>
<td>39</td>
</tr>
</tbody>
</table>
recently used to automate the pipetting steps involved in extraction procedures but the most recent improvements are directed toward the use of magnetic particles to clean up or extract the sample prior to analysis [39,124–127].

3.2 Novel Assays

The first point-of-care test (POCT) for the quantitative measurement of 25OH Vitamin D was approved by the FDA in 2013 [116]. This test is based on magnetic particles and relies on chemiluminescence for the detection part. The difference with the classical CLIA assays is its innovative single patient format. The reagents are contained in a pouch and a small instrument activates the different parts of the pouch to mix and transfer the reagents from one section of the device to another. The reading is also performed within the instrument. This system allows the quantitative measurement of 25OH Vitamin D in, e.g., physician office labs in only 12 min. Although 25OH Vitamin D is not an urgency marker is the case for cardiac markers, for example, a POCT enables the physician to directly prescribe or adjust Vitamin D supplementation without the need for an additional visit by the patient.

Qualitative assays have also been developed and are based on lateral flow formats and on visual detection based on cutoff values [128]. Although they seem attractive and can be used on either serum/plasma or whole blood, none of them has gone through clinical validation and CE or FDA registration so far.

Vitamin D metabolites are traditionally assessed in serum or plasma but saliva is another interesting matrix that is currently attracting interest from the research community [13–16]. The main advantage of saliva is the non-invasive collection method that allows a nonphysician to collect samples. It opens the field to paramedical professionals and to large studies in collectivities such as schools or sport centers, for example. There are two main challenges associated with the measurement of 25OH Vitamin D in saliva. First, as the expected concentration of 25OH Vitamin D in saliva is about 5000 times lower than in whole blood, any traces of blood that might be present in saliva will automatically false the results. Particular attention must, therefore, be paid at the collection stage in order to obtain a clean saliva sample. Second, the concentration of 25OH Vitamin D in saliva is very low and in the range of 2–15 pg/mL. Very sensitive assays have to be developed to be able to measure such low concentrations of this molecule.
Furthermore, as the binding proteins are not capable of crossing the blood to saliva membrane, the concentration of 25OH Vitamin D in saliva is expected to reflect the concentration of the free metabolite in blood. As seen in previous chapters free 25OH Vitamin D does not systematically correlate to total 25OH Vitamin D and this must be taken into consideration when envisaging the measurement of 25OH Vitamin D in saliva.

Free 25OH Vitamin D is certainly one of the most important topic for the coming years. Recent publications are showing that measuring free 25OH Vitamin D might be a better indicator of Vitamin D action in several conditions such as pregnancy and liver disease. Further research must be conducted to fully evaluate the potential of this marker in clinical conditions. Although a direct measurement procedure already exists, work is done on the development of accurate VDBP methods, mainly by LC–MS, to allow for good calculations of this free metabolite.

4. CONCLUSION

Vitamin D deficiency is a worldwide problem and both the research community and the general public are more and more aware of the phenomenon. This has led to an explosion of the number of Vitamin D tests over the past 5 years and from three or four commercial assays the field has evolved to certainly more than 20 assays nowadays. With such an evolution the need for standardization has rapidly emerged. This gap has been partially closed, thanks to the Vitamin D Standardization Program but many efforts remain to be done in order to harmonize all existing and new assays.

The measurement of 25OH Vitamin D has moved from a RIA and ELISA mix to a field largely dominated by CLIA methods, mostly thanks to automation and short turnaround times. LC–MS, considered as the Golden Standard for the measurement of 25OH Vitamin D, is also widely present in the United States and in central Europe. Although modern immunoassays are more and more based on monoclonal antibodies, many of them still exhibit some cross–reactivity with one of the Vitamin D metabolite, the 24,25(OH)₂ Vitamin D. Whether this cross–reactivity is only present in spiked samples or is also present in patient samples is not clear yet. Another point of attention lies in the method that is used to release 25OH Vitamin D from its binding proteins. High concentrations of VDBP might interfere with the assay formulations and lead to biased results. In addition to its technical complexity LC–MS can confound C3-epi-25OH Vitamin D with 25OH Vitamin D, providing falsely elevated results in pediatric samples.
Further developments should be aimed at solving these issues and bring standardized, reproducible, and accurate assays in the laboratories.

The measurement of 1,25(OH)\(_2\) Vitamin D is mainly requested for samples from patients undergoing dialysis. Until recently it was only performed by either RIA or ELISA methods. LC–MS has appeared but in a limited number of laboratories due to the very high complexity of the method. Two CLIA methods have recently become available and allow a fully automated testing of this Vitamin D metabolite. Many work remains to do in this field in terms of standardization to a RMP, cross-reactivity to the D2 form, and simplification of the methods.

Besides the routine testing of 25OH Vitamin D and 1,25(OH)\(_2\) Vitamin D, research assays have been developed for the measurement of 24,25(OH)\(_2\) Vitamin D, and for free and bioavailable 25OH Vitamin D, which are thought to be superior markers of Vitamin D actions in case of low or high VDBP concentrations.

The field of Vitamin D testing has tremendously evolved the past 5 years and will continue as such for the near and far future. Existing assays must be optimized and novel assays have to be developed to enable the researchers to better understand the complex Vitamin D physiology and metabolism and to define the future of Vitamin D testing in clinical laboratories.

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