Vitamin D Deficiency: Causes & Treatment

Chapter 3

Analytical Aspects of Vitamin D

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Abstract

This chapter deals with vitamin D and its metabolites from the point of view of the steps involved in their analytical determination. Thus, after a brief description of the characteristics and metabolism of vitamin D to introduce the target metabolites, the analytical aspects of these compounds are addressed. First, we discuss the suitability of the types of samples (*e.g.,* fresh and dry blood, urine, saliva, cerebrospinal fluid) in which vitamin D and its metabolites are determined; then, the stability of the most used sample (blood as both serum and plasma) under different storage conditions is considered in the light of the different metabolites to be measured. The half-life and levels in blood of these compounds, the sources and supplementation with vitamin D, as well as the role of vitamin D and its metabolites in human health are also matters of this chapter prior to discuss in depth the sample preparation and derivatization steps and emphasize a key aspect as sample volume. The description and discussion of the analytical methods for determination of vitamin D and its metabolites encompass, as the most important, immunoassays methods and mainly those involving chromatographic separation prior to detection, with mass spectrometry and the gold technique for identification and determination.

Abbreviations: ACN: Acetonitrile; AD: Ampliflex™ Diene; ALTM: All-laboratory trimmed mean; APCI: Atmospheric pressure chemical ionization; CPBA: Competitive protein binding; CV: Coefficient of variation; DAD: Diode array detector; DBP: Vitamin D binding protein; DBS: Dried blood spots; DEQAS: International Vitamin D External Quality Assurance Scheme; FI: Flow-injection; GC: Gas chromatography; IAE: Immunoaffinity extraction; LC: Liquid chromatography; LLE: Liquid–liquid extraction; LLOQ: Lower limit of quantitation; NIST: National Institute of Technology; PTAD: 4-phenyl-1,2,4-triazole-3,5-dione; QqQ-MS: triple quad mass spectrometry detector; RIA: Radioimmunoassay; RRB: Radioreceptor binding; SIL-IS: Stable isotopically labeled internal standard; SPE: Solid-phase extraction; SRM: Selected reaction monitoring; UV-B: Ultraviolet B

1. Vitamin D: Characteristics and Metabolism

Vitamin D is a prohormone that comes in many forms, but the two major physiologically relevant ones are vitamin D_3 (cholecalciferol) and vitamin D_2 (ergocalciferol). Technically, vitamin D is classified among secosteroids in which one of the rings has been broken **(Figure 1).** As derived from a steroid, the structure of vitamin D retains its numbering from its parent compound cholesterol: the 9,10 carbon–carbon bond of ring B is broken, as indicated by the inclusion of "9,10-*seco*" in the official nomenclature. The configuration of the double bonds are notated *E* for "entgegen" or *trans*, and *Z* for "zuzammen" or *cis* [1]. Thus, the official name of vitamin D_3, by relation to cholesterol, is 9,10-*seco* (5Z,7E)-5,7,10(19) cholestatriene-3β-ol; while the official name of vitamin D_2 is 9,10-s*eco* (5Z,7E)-5,7,10(19),22-ergostatetraiene-3β-ol. The differences between both are a double bond between C22 and C23, and a methyl group on C24 for vitamin D_2.

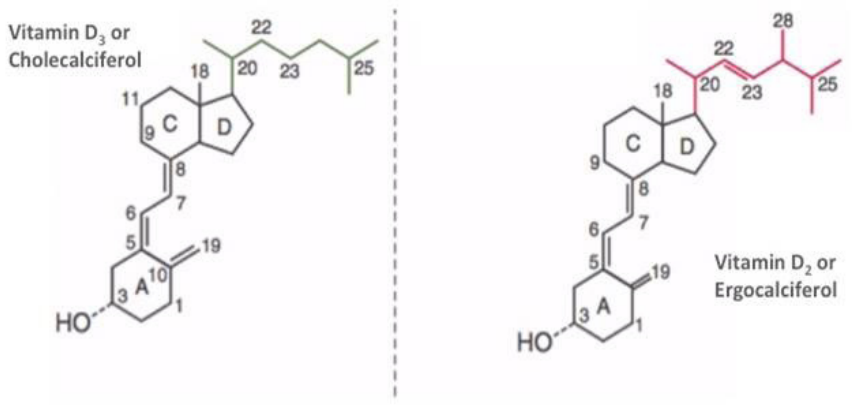

Figure 1: Important nutritional forms of vitamin D.

Vitamin D_3, the naturally occurring form, originates from dermal synthesis by conversion of 7-dehydrocholesterol, a precursor of cholesterol, into pre-vitamin D_3 under ultraviolet UV-B radiation, on the plasmatic membrane of skin cells. This process is followed by fast thermal isomerization to vitamin D_3 **(Figure 2)**. Approximately 50% of pre-vitamin D is converted into vitamin D in 2 h, formed in the cell membrane and then transported to the extracellular space. From this, vitamin D is transported in blood bound mainly by the transporter vitamin D binding protein DBP to reach the liver. There, vitamin D-25-hydroxylasa of cytochrome P450 produces the 25-hydroxylation in the liver to yield the main circulating form, 25(OH)D. D-25-hydroxylasa is not strictly regulated in the liver; therefore, an increase of cutaneous production of vitamin D or its intake increase the level of circulating 25(OH)D —both 25(OH)D_3 and

25(OH)D_2. Therefore, the joint measure of both monohydroxylated vitamin D metabolites is used to determine the status of vitamin D.

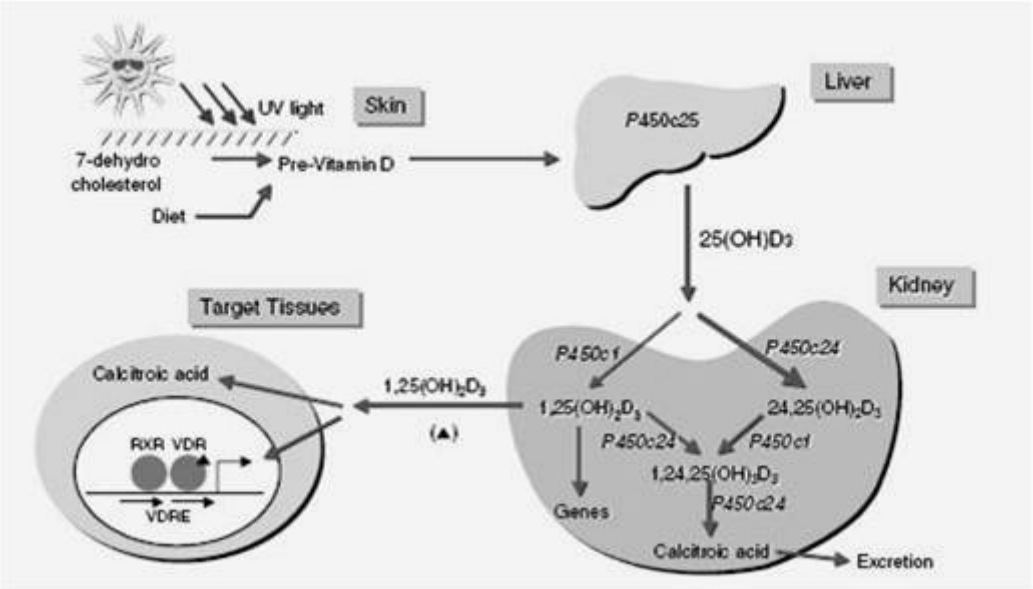

Figure 2: Activation and molecular pathways for vitamin D: integrative schematic synthesis, metabolism and molecular action.

Further 1α-hydroxylation of 25(OH)D both in the kidney and extrarenal sites gives place to the hormonal form, 1,25(OH)$_2$D, which mediates its pleiotropic effects through the ubiquitous vitamin D receptor that binds to vitamin D response elements in target genes to regulate their transcription [2]. 1,25(OH)$_2$D has potent antiproliferative and cell differentiation-inducing activities in addition to its role in calcium homeostasis [3]. The inactivation of vitamin D is carried out by side chain oxidation by the mitochondrial 24-hydroxylase, which catalyzes the conversion of both 25(OH)D_3 and 1,25(OH)$_2D_3$ into a series of 24- and 23-hydroxylated products targeted for excretion, culminating in the water-soluble biliary metabolite 26,23-lactone and calcitroic acid [4]. C3-epimerization is a second biochemical pathway, via which the major vitamin D metabolites —25(OH)D_2, 24,25(OH)$_2D_3$ and 1,25(OH)$_2D_3$— are converted to their respective epimeric forms and are further metabolized through the C-24 oxidation pathway [5].

Even though 1,25(OH)$_2$D is in fact the biologically active form of vitamin D, serum 1,25(OH)$_2$D provides no information about vitamin D status and is often normal or even elevated due to secondary hyperparathyroidism associated with vitamin D deficiency. On the contrary, 24,25(OH)$_2D_3$ is the major circulating dihydroxylatedvitamin D metabolite in human serum with concentrations between 0.7–40 nmol/L [6,7]. Serum 24,25(OH)$_2D_3$ concentrations show a strong positive correlation with serum 25(OH)D_3 levels, and are approximately 10% of 25(OH)D_3 levels [7–14].

2. Analytical Aspects of Vitamin D

This section involves subjects such as the clinical samples used for vitamin D determination, the stability of the most frequently used samples, half-life and normal levels in

humans of vitamin D and its metabolites, main sources of vitamin D and supplements on this vitamin, as well as its role in human health.

2.1. Clinical samples for determination of vitamin D

Biological matrices for determination of vitamin D and its metabolites in human include dried blood spots (DBS) [15–22], urine [23], saliva [24], cerebrospinal fluid [25], and mainly blood.

DBS collection is a minimally invasive sampling to obtain blood samples on cards or filter paper. Therefore, it is a practical way to screen for vitamin D deficiencies in large epidemiological studies or in at risk populations where venipuncture is impractical, including pediatric populations, rural clinics, and developing countries, or where blood volume is limited as in neonates [19]. Calibration of DBS analysis is difficult owing to the absence of blank samples in the case of endogenous analytes, and the fact that current reference analytics is performed using plasma or serum samples and not dried blood [20].

Measurement of vitamin D in saliva is interesting from the perspective that it is likely to reflect the free, non-protein-bound hormone in plasma [26], and may serve as a better indicator of vitamin D status [27]. Concentrations of salivary $25(OH)D_3$, however, are at the picomolar ranges, less than one-thousandth of that in plasma, and require sensitive assay technology for measurement [28]. A good correlation was found between the serum and salivary $25(OH)D_3$ levels [24]

Urine is a more complicate sample for vitamin D analysis as most of its metabolites are conjugated; therefore, in addition to volume normalization, if required, sample preparation involves hydrolysis (usually catalyzed by β-glucuronidase, followed sometimes by derivatization). Ogawa *et al.* found the amount of $24,25(OH)_2D_3$ in urine 2–3 times greater than that of $25(OH)D_3$, contrary to the 1:10 ratio of $24,25(OH)_2D_3$ to $25(OH)D_3$ in serum [23]. Sulfate conjugated vitamin D metabolite $25(OH)D_3$ 3-sulfate has also been found in human plasma at levels similar to or higher than that of $25(OH)D_3$ in adults or infants, respectively, but lower in preterm infants [29]. This conjugate has not been proven to have significant biological activity [30].

Cerebrospinal fluid from multiple sclerosis patients was used to study the controversial information on the effect of vitamin D metabolites on the disease. The concentration of 25(OH)D in this biofluid from the target patients was not significantly different as compared with patients with other inflammatory neurological diseases, but the cerebrospinal fluid/serum ratio was significantly lower in multiple sclerosis patients [25]. The authors of this study stated that the results they obtained do not support that 25(OH)D is actively transported to the cerebrospinal fluid, or that the cerebrospinal fluid or serum levels or their ratio exert a

major impact on multiple sclerosis activity. A comprehensive metabolomics study of vitamin D metabolites in multiple sclerosis patients has not been developed so far [31].

Blood, either as serum or plasma is, with a high difference, the most used biofluid for clinical studies of vitamin D and its metabolites in such a way that the reference values for the status of vitamin D are established in one of them.

Blood (as serum or plasma) has been the clinical sample preferred by the authors' research team for development of methods for determination of vitamin D and its metabolites. The team has worked on the metabolism of vitamin D since 1997, when metabolomics had not been defined as such omics discipline, and published 7 articles between 1997–1999 dealing with methods to improve the determination of vitamin D and metabolites using plasma as clinical sample [32–38]. Also a review on the state-of-the-art and trends on the determination of vitamin D [39] was published by the authors. Further studies on vitamin D metabolites developed by the research team (between 2007 and 2013) were based on serum as clinical sample, and mainly devoted to reduce the sample size, automation of sample preparation, improvement of sensitivity and cross-sectional studies [40-48]. More recently, the team has developed a study on the suitability of serum or plasma for the determination of vitamin D and its key mono- and dihydroxymetabolites [49].

2.2. Stability of serum/plasma samples for analysis of vitamin D

Vitamin D and its metabolites have traditionally been considered unstable compounds sensitive to light and temperature [50]; therefore, studies on their stability have been developed, but not in depth. A study published in 2004 was devoted only to the 25(OH)D metabolite, which was determined by a chemiluminescence immunoassay method in serum and plasma samples both fresh and frozen [51]. Comparison of the results showed *p* values greater than 0.7, indicating no significant difference between fresh and frozen samples, the latter subjected to five freeze–thaw cycles; thus demonstrating that the samples did not need to be frozen if they are assayed within 5 days from sampling. The equivalent cross-reactivity of 25(OH)D_2 and 25(OH)D_3 in the target immunoassay used was also demonstrated. Cross-reactivity to the dihydroxy vitamin D metabolites was accepted, but without clinical concern due to the approximately 1000-fold concentration difference between these metabolites and the 25(OH)D in the circulation. As the authors stated, concentrations of 1,25$(OH)_2$D at five times normal values would contribute less than 0.05 nmol/L to the measured 25(OH)D concentration. Thus, pathological 1,25$(OH)_2$D concentrations would be expected to contribute less than 0.1% to the overall imprecision. A further study in serum involving only the 25(OH)D metabolite and RIA analysis showed stability of this compound at –25 ºC in storage between 6 and 24 years [52].

In a more recent study on stability of the 25(OH)D metabolite in serum ,the levels determined by LC–MS/MS were compared with those obtained by conventional radioimmunoassay

(RIA) –no distinction between 25(OH)D_3 and 25(OH)D_2— [53]. Note that 1,25(OH)$_2$D, less concentrate than the monohydroxy metabolites, was not determined. The values obtained by the RIA method exhibited a mean bias of about 8.35 ng/mL, most likely as a result of cross-reaction of the antibody with low-abundant metabolites, including 24,25(OH)$_2D_3$. Various preanalytical factors, such as long sample sitting prior to serum separation, repeated freeze–thaw cycles, and the presence of anticoagulants had no significant effects on the determinations.

A more comprehensive stability study was developed by the authors' team using serum and plasma samples subjected to: (1) room temperature, 25 ºC preserved from sunlight; (2) refrigeration, 6 ºC, and freezers, (3) at –20 ºC and, (4) at –80 ºC. The subsequent monitoring of vitamin D and its mono- and dihydroxymetabolites, the influence of freeze/thaw cycles on the stability of the target compounds, as well as the behavior of lyophilized serum and plasma have been the subject of the study [54].

2.3. Half-life and levels of vitamin D metabolites in humans

The parent sterol vitamin D has a half-life close to 24 h [55], relatively short as compared with 25(OH)D, which has a half-life of 2–3 weeks [56,57]. Therefore, 25(OH)D measurement is a better indicator of vitamin D storage, whether obtained from sunlight (UV exposure) or dietary sources. The most potent physiologically active circulating metabolite produced by humans is 1,25(OH)$_2$D, which has a half-life of 4–8 h [58–60]. While 25(OH)D circulates at the nmol/L concentrations, 1,25(OH)$_2$D is present at the pmol/L concentrations; at a 1000-fold lower concentration than 25(OH)D (with the reference interval for healthy adults being 38–134 pmol/L [61]), which means that this dihydroxymetabolite represents the greater challenge in assay development.

The levels of vitamin D metabolites that define normal, abnormal or pathological values in adult humans are established with respect to the circulating form. There is virtually unanimous agreement that a serum level of 25(OH)D less than 30 nmol/L defines vitamin D deficiency, but there are different opinions regarding the 25(OH)D levels that define vitamin D insufficiency which may differ from 50 nmol/L [62] to 75 nmol/L [63]. The Institute of Medicine recommends that deficiency corresponds to <30 nmol/L, and places a person risk relative to bone health; 30–50 nmol/L places some, but not all, persons at risk for inadequacy, which can be considered as insufficiency; while sufficiency (adequate) is established for ≥50 nmol/L that meets the needs of 97.5 % of the population. About 1 billion people worldwide are estimated to have 25(OH)D levels of less than 75 nmol/L [64]. The Institute of Medicine also states that levels higher than 75 nmol/L are not consistently associated with increased benefit [65]. **Table 1** summarizes the levels of both vitamin D and 25(OH)D as expressed by K. Poongkodi [66] including extreme values.

Table 1. Levels of vitamin D and 25(OH)D which define normal, abnormal and pathological values in serum from human adults [66].

Status	25(OH)D, ng/mL	Vitamin D, nmol/L
Severe Deficiency	<10	<25
Deficiency	<20	<50
Insufficiency	21 – 29	50 – 74
Sufficiency	30 – 100	75 – 250
Optimal	30 – 60	75 – 150
Toxic	>150	>375

It has also been proposed that free or bio-available 25(OH)D provides a better assessment of vitamin D sufficiency than total 25(OH)D [27]. Free or bio-available 25(OH)D can be calculated taking measured DBP and albumin into account [67] or can be directly measured by immunoassay [68]. The 24,25$(OH)_2D_3$ to 25$(OH)D_3$ ratio may also be a predictor of serum 25$(OH)D_3$ response to vitamin D_3 supplementation [11,14,69].

Differentiation between the concentration of 25(OH)D and its epimer 3-epi-25(OH) D in infants is of interest in this population as studies in up to 1 year of age have shown significant 3-epi-25(OH)D concentrations as high as 200 nmol/L with relative contribution to total 25(OH)D as high as 55% [70–76]. This behavior can be attributed to either transference of the epimer in the utero or to postnatal formation.

2.4. Sources of and supplementation with vitamin D

Vitamin D is derived from two major sources in humans, with approximately 80–90% produced on the skin resulting in cholecalciferol (D_3), and the other 20% is derived from dietary sources, which can be animal cholecalciferol (D_3) or plant derived ergocalciferol (D_2). The latter originates from the yeast and plant sterol ergosterol and is obtained from diet or supplements.

Small amounts of vitamin D can also be obtained by nutritional intake of either vitamin D_3 or by foods fortified with vitamin D_2. Both forms undergo a substrate-dependent liver hydroxylation to 25-hydroxyvitamin D [25$(OH)D_2$ and 25$(OH)D_3$], as stated in **Figure 2**.The perceived conception that vitamin D supplementation can prevent, improve or cure chronic disorders has caused over the last years a massive rise in demand for measurement of 25(OH) D in blood as surrogate marker of vitamin D status.

There is much interest in intermittent dosing for patient convenience, and long term adherence, but caution is warranted when using supraphysiological bolus doses as these may be counterproductive leading to transient vitamin D intoxication in the immediate post-dosing period [77–79] or adverse effects in the longer term [80,81]. There are not conclusive results

on vitamin D supplementation. Thus, interventional studies on vitamin D supplementation (1.200 IU vitamin D per day) in patients with Parkinson disease showed no conclusive results when compared with individuals who received placebo during the same period [82]. On the contrary, 5 studies of vitamin D supplementation in rodents on the effect of vitamin D therapy on substantianigra dopamine neurons resulted in: one study with a significantly higher dopamine level and two a significantly higher concentration of the enzyme promoting conversion to dopamine (tyrosine hydroxylase), in the substantianigra of the vitamin D supplemented rodent group [83–85]. One of these studies also demonstrated that oxidative injury of the substantianigra was significantly lower after vitamin D supplementation [83]. This result was in line with another study reporting higher neuronal survival in the substantianigra when $1,25(OH)_2D$ was injected [86]. However, very high concentrations of $1,25(OH)_2D$ enhanced neurotoxity, as also found in Klotho-insufficient mice that resemble human aging and exhibit abnormal levels of serum $1,25(OH)_2D$ caused by abnormally high vitamin D metabolism in the kidney [86,87].

In infant studies, vitamin D supplementation (2.000 IU/day) in the first year of life increased the risk of developing atopy, allergic rhinitis, and asthma late in life [88].

2.5. The role of vitamin D and its metabolites in human health

Over the past decade, more than 1600 studies have been conducted on vitamin D, and more than half of them are cohort or observational studies demonstrating an association between deficits in vitamin D and a litany of acute and chronic disorders (cardiovascular disease, cancer, diabetes, fractures, depression and respiratory tract infections, to name a few) [89]. These findings have fueled the hypothesis that vitamin D supplementation—a widely available, low-cost and mostly harmless intervention—might treat or even prevent these disorders. Association, however, is not causation.

At present, evidence supports vitamin D supplementation to help prevent fractures (particularly if given with calcium), and possibly to prevent falls and slightly reduce mortality (particularly in older patients [>70 years of age]). No other effects have been proven. For many other conditions, the evidence for vitamin D supplementation is plagued by the use of small, poor-quality trials. Lastly, testing of 25(OH)D levels in the general population is not necessary, and very high doses should be avoided.

Clinical interest in Vitamin and its purported roles not only in calcium and bone metabolism but in several other medical conditions (diabetes, cardiovascular disease, multiple sclerosis, cancer, psychiatric disorders, neuro-muscular disease) has led to a surge in laboratory requests for 25(OH)D and $1,25(OH)_2D$ measurement, but also their ratio and ratios of other vitamin D metabolites. The search for vitamin D metabolites-based biomarkers that could be associated to given diseases is other of the open fields for interesting research, which the authors' team

has contributed. First, a proper use of the metabolomics in the field of vitamin D as diseases biomarkers started with the publication of a review on "the analytical process to search for metabolomics biomarkers", to establish the basis for use of the proper analytical steps in each case [90]. Other contributions have constituted calls of attention on the unclarified action of the different metabolites of vitamin D on multiple sclerosis and the role of metabolomics to clarify the vitamin D–multiple sclerosis relationship [31,91].

3. Sample Preparation for Determination of Vitamin D and its Metabolites

3.1. Sample preparation

Sample preparation for determination of vitamin D in the clinical field has been the workhorse of most analytical chemists working in this field, who have looked for interferents removal, automation and preconcentration steps to decrease sample volume as much as possible, increase sensitivity, selectivity and the number of analytes to be determined, and shorten the analysis time. Therefore, traditional sample preparation steps have consisted of:

(1) Deproteination or removal of protein and related macromolecules, a common step in dealing with serum or plasma samples. The step involves mixing equal volumes of sample and precipitant reagent such as acetonitrile (ACN) [92–98], ammonium sulfate [99,100] or ethanol [101,102]. This is the only preparation step prior to selective quantitation (*e.g.*, RIA).

(2) Saponification, mainly used with samples containing high contents of lipids such as infant formulae [102,103], enriched milk [104–107], eggs [108], fish oil [108–111] or margarine [112]. This step substitutes deproteination and is followed by liquid–liquid extraction (LLE). Saponification and subsequent removal of lipids involve losses of vitamin D and its metabolites by dragging; losses that have been traditionally evaluated by a recovery study using radioactive isotopes.

(3) LLE constitutes an alternative to the two previous sample preparation steps. It can be simple or multiple LLE. The most general manual procedure has been that of Bligh and Dyer [113].

(4) Solid-phase extraction (SPE), used since the earliest methods for quantitation of vitamin D and its metabolites, underwent a remarkable expansion with commercialization of SPE cartridges, which virtually substituted manual column packing.

The improvement of sample preparation achieved by SPE can be summarized in: lower amount of neutral lipids in the extract as compared with LLE; higher protection of the equipment used for subsequent individual separation (LC or GC) or by direct quantitation by competitive protein binding (CPBA) radioreceptor binding (RRB) or RIA; effective decrease in costs in terms of cartridges and solvents; high preconcentration factors by final elution

with small eluent volumes; and, availability for selective separation of the analytes based on polarity differences.

Nevertheless, SPE also involves some drawbacks such as: necessity for calibration procedures to minimize the variability among commercial cartridges; potential introduction of contaminants that can remain in the sorbent and might be eluted in subsequent steps; excess of confidence in the cleanup capacity of the procedures, with absence of checking and control steps.

(5) Liquid chromatography preparation as step previous to individual chromatographic separation. Molecular exclusion [114,115], solid–liquid partitioning either by normal [116–118] or reverse-phase chromatography [114,119,120] or a combination of them [115,119,120] have been used prior to RIA [121] or CPBA [94].

An overview of the contributions to sample preparation for determination of vitamin D and its metabolites by the authors' research team are as follows:

A first contact of the authors' team by the last decade of the XX century with its new research line on vitamin D was to improve a previous sample preparation SPE procedure for plasma found in the literature [122]. Keeping protein precipitation by ACN, the SPE step was improved by more efficient mixtures used for washing and elution from two subsequent cartridges (Bond-Elut C18 and Bond-Elut silica)[32]. A drastic decrease of interferents (a cleaner LC–UV-chromatogram) that allowed the determination of 24,25$(OH)_2$D, 1,25$(OH)_2$D, in addition to 25(OH)D, the only determined in the previous procedure [122].

An on-line coupling of a flow-injection (FI) manifold to the loop of the injection valve of an LC–UV detector arrangement allowed partial automation of the SPE step by locating a C18 minicolumn in the loop of the FI injection valve and selecting sequential passage through it of conditioning solution, sample, washing solution, and eluant [33]. The continuous method provided as main advantages a single and miniaturized SPE (smaller amount of sorbent), reusability of the minicolumn, drastic preconcentration factor and simple automation. The method thus developed was improved by a more effective protein precipitation by changing ACN to isopropanol, then compared advantageously with an RIA method for 25(OH)D [34]. The use of an aminopropyl-silica sorbent and the same continuous arrangement showed the following advantages provided by this polar sorbent instead of the nonpolar C18 sorbent [35]: lower detection and quantitation limits and better CV % values and recoveries than the previous methods. A new application of the arrangement allowed the determination of vitamins D_2, D_3, K_1 and K_3, and also the 25$(OH)D_3$, 24,25$(OH)_2D_3$ and 1,25$(OH)_2D_3$ metabolites [36]. Other improvements involved an LLE step prior to SPE and the same continuous system, including postcolumn derivatization and changing the UV-chromatographic detector to a conventional fluorimetric detector (sensitivity increased about 50 times with respect to previous methods)

[37], or a laser-induced fluorescence detector (sensitivity 10 times higher than with the conventional fluorimetric detector) [38].

Almost all subsequent developments of the team in this research line during the XXI century have involved commercial automated high-pressure SPE stations (Prospekt1, Prospekt2 or Symbiosis Pharma, all from Spark-Holland) on-line connected to the chromatograph in such a way that the mobile phase acts as eluent, thus inserting in the chromatographic column the total amount of the compounds retained in the CN cartridge. The first contribution —for determination of 25(OH)D_3, 24,25$(OH)_2D_3$ and 1,25$(OH)_2D_3$ — involved a first manual LLE step followed by the SPE step by Prospekt1 and the final LC–DAD (diode array detector) step. A cleaner chromatogram, better reproducibility and shorter analysis time were thus achieved [40]. A subsequent development allowed total automation of the method by the use of a robotic workstation to develop the LLE step. In this way vitamins A and E, and the 25(OH)D_3, 24,25$(OH)_2D_3$ metabolites were determined (the wavelength of maximum absorption for each analyte was selected taking advantage of the DAD) [41]. A further application allowed vitamins A and E, and the 25(OH)D_3, 24,25$(OH)_2D_3$ metabolites to be determined in the normality range of these compounds in healthy individuals within the 18–80 year-old interval [42]. Avoidance of the LLE step and use of the serum sample mixed with 0.5 mL of ACN containing sodium dodecyl sulfate allowed direct insertion in the Prospekt1 system and chromatographic separation–DAD determination of fat soluble vitamins A, D_2, D_3, α- and δ-tocopherol, and 25(OH)D_3 in 20 min [43].

A key improvement of the methods for the target compounds was achieved by change the DAD to a triple quad mass spectrometry (QqQ-MS) detector. The first application of this detector, always coupled to LC equipment, involved manual sample preparation consisting of protein precipitation with methanol, LLE with hexane, evaporation of the extractant and reconstitution of the residue with methanol prior to injection in the chromatograph for separation of vitamins A, K_1, K_2, D_2, D_3 and E (as α- and δ-tocopherol), and the 25(OH) D_2, 25(OH)D_3, and 1,25$(OH)_2D_3$ metabolites [44]. The use of the commercial SPE station Prospekt2 on-line connected to the LC–QqQ-MS arrangement allowed to work with only 0.2 mL of serum sample injected (after protein precipitation) into the sample loop of the SPE station provided with Hysphere cartridges automatically conditioned. The analysis time was 20 min [45]. The features of the method made feasible its application to cross-sectional studies [47,48]. The most recent innovation in the equipment for sample preparation used by the authors' team was substitution of the Prospekt 2 by the Symbiosis Pharma model, designed for high sampling throughput with samples maintenance at 4 ºC, with subsequent in depth optimization of volumes and times for the equilibration, sorption and elution steps [49].

3.2. Derivatization

Derivatization steps based on dehydration [123–126], cycloaddition [127–130], silanization [116–118,131,132], or charge-transfer complex formation [133] have been implemented as a pre- or postcolumn step in either LC or GC.

(1) Dehydration reactions occur by exposure of vitamin D and its metabolites to high temperature. The non-specific reaction produces B-ring cyclation to yield pyro and isopyro isomers. The number of isomeric structures thus produced significantly complicates subsequent individual separation and identification. Cyclation constitutes a hard limitation for development of methods involving GC. Selective cyclation to obtain given products [122] or more thermostable products by isotachysterol formation [134–136] has been reported as a step prior to GC–MS [122].

(2) Diels–Alder cycloaddition contributes to enhancing sensitivity and selectivity in the determination of vitamin D and its metabolites, a reaction that can be developed by very different reagents and has been implemented both in pre- and postcolumn location in LC–MS methods [119,127–130].

(3) Silanization reactions have been used to minimize unspecific adsorption of vitamin D and its metabolites in GC mainly owing to the presence of hydroxyl groups on these molecules. A number of silane-derivatives have been used with this aim [93–100,137–139].

(4) Charge transfer-complex formation has also been implemented, mainly for the determination of vitamin D in pharmaceuticals [133].

Derivatization reactions have been applied by the authors' research team, always in poscolumn location, with the aim of increasing sensitivity as the necessary selectivity was supplied by LC separation. A Diels–Alder reaction using 4-phenyl-1,2,4-triazole-3,5-dione (PTAD) formed an adduct with maximum absorption at 337 nm, which increased the sensitivity 5-times with respect to the method without derivatization [36]. A dehydration method favored by a strong-acid medium was also postcolumn developed which improved fluorimetric detection (with conventional [37] or laser-induced detectors [38]).

3.3. Sample volume

The sample volume of either serum or plasma required for determination of vitamin D metabolites has experienced a spectacular decrease thanks to the improvement of sample preparation steps, and increased sensitivity of the detectors used (mainly MS detectors). Thus, the isotope dilution-mass fragmentography assay for $1,25(OH)_2D$ published in 1979 [140] required 20 mL of serum subjected to LLE with a chloroform–methanol mixture after addition of [26-$^2H_3$]-$1,25(OH)_2D_3$, and purification by LC. Then, the metabolite in the purified material

was converted into the trimethylsilyl derivative and analyzed by GC–MS. The lower limit of quantitation (LLOQ) was 13 pmol/L (5 pg/mL), with a CV of 5%; but the large sample volume limited the general applicability of the assay. The evolution of analytical equipment and sample preparation devices led to a method published in 2010, based on LC–MS/MS [141] by which both $1,25(OH)_2D_2$ and $1,25(OH)_2D_3$ could be measured in 30 μL of sample. An LLOQ for $1,25(OH)_2D_3$ of 15 ng/L (36 pmol/L), a CV of 5–15 % across physiological concentrations, and a total run time per sample of 30 min supported the excellent features of the method. Sample preparation involved a complex on-line process using a perfusion column, followed by a chain of two monolithic columns to clean and enrich the sample prior to LC–MS/MS analysis without derivatization. A recently published method uses a 20 μL serum sample volume for determination of four vitamin D metabolites —*viz.* $25(OH)D_3$, 3-epi-$25(OH)D_3$, $25(OH)D_2$, and $24,25(OH)_2D_3$; with LOQ of 0.091, 0.020, 0.013, and 0.024 ng/mL, respectively. The very long sample preparation process involved SPE on wells plates, incubation, evaporation, and derivatization for 1 h prior to injection into the LC–MS/MS system [53].

In the studies developed by the authors' research team the maximum sample volume used was 2 mL [32–38,40,41], then reduced to 1 mL after exhaustive optimization [42,44,46], and to 0.5 mL by eliminating the LLE step [43], and, finally 0.2 mL when the Prospekt2 workstation was used. In our more recent research the sample volume has been 240 μL in all instances as a compromise between the required sensitivity for determination of the more diluted metabolites and the possibility of obtaining samples from patients in critical state [49,142,143].

3.4. Analysis time

A sometimes crucial analytical parameter as analysis time is very difficult to be deduced from most of the publications on this subject. A number of published methods for determination of vitamin D and its metabolites only specific the time required for the chromatographic–detection step, despite obtainment of the analytical sample [144] has involved deproteination, centrifugation, LLE, evaporation of the extractant to dryness one or several times, derivatization with enough completion requiring 1 h or even more, etc.

In the recent research developed by the authors, once the method for determination of vitamin D and its metabolites was enough validated for massive application, the time required for analysis was 15 min, achieved by overlapping of the chromatographic–detection steps of one sample with the SPE of the next [49,142,143]. The only pretreatment after sample thawing is centrifugation/filtration prior to location in the thermostated autosampler.

4. Methods for Determination of Vitamin D and its Metabolites

Methods for vitamin D metabolite measurement can be divided into two main groups: immunochemical methods and those based on liquid chromatography separation, mainly with

MS detection.

4.1. Immunoassay methods

Immunoassays dominate 25(OH)D testing (90%) in clinical laboratories and have evolved from laborious manual RIA formats to fully automated assays on random access analyzer platforms. These automated immunoassays are mainly based on a competitive principle (Ab or protein binding formats) but recently, a promising sandwich assay based on the metatype antibody principle has become available [145,146]. Automated immunoassays seem attractive for reasons of high throughput capabilities. However, they have some major shortcomings such as limited sensitivity and dynamic range, difficulties in DBP displacement, non equimolar detection of $25(OH)D_2$ and $25(OH)D_3$, interferences from heterophilic antibodies, and from gel and clot activator in blood collection tubes [147], lack of adequate standardization, and cross-reactivity towards other major circulating vitamin D metabolites, $24,25(OH)_2D_3$ being the most abundant. Particularly, in specific populations with altered DBP status, as in dialysis patients, intensive care unit patients and in pregnancy, immunoassays show remarkable differences when compared to LC–MS/MS which have been attributed to inefficient release of 25(OH)D from DBP [148–150].

Immunoassay manufacturers correct for $24,25(OH)_2D_3$ cross-reactivity in a fixed manner, which may lead to overcorrection, and to falsely low 25(OH)D as a result, in patients with lowered $24,25(OH)_2D_3$ levels, as in chronic kidney disease [151,152].

The most commonly used methods for $1,25(OH)_2D_3$ quantitation are competitive RIA with ^{125}I as the radio-label, or enzyme immunoassays that require extensive sample purification to minimize contribution of other vitamin D metabolites [153]. In vitamin D intoxication, elevated levels of 25(OH)D can interfere with $1,25(OH)_2D_3$ measurement using RIA owing to 25(OH)D cross-reactivity of the $1,25(OH)_2D$ antibody [154–156]. More recently, fully automated chemiluminescence immunoassays that accurately and precisely measure $1,25(OH)_2D$ have become available. They show good correlation with well validated LC–MS/MS assays [157–162].

An area of concern in relation to immunoassays is the variability in the detection of $25(OH)D_2$. Some assays claim to have 100% cross-reactivity with exogenously added $25(OH)D_2$ and $25(OH)D_3$ and are therefore equipotent for the measurement of the two metabolites. Other assay manufacturers admit to lower cross-reactivity with exogenous $25(OH)D_2$ (75% [kit insert from IDS, Boldon, UK], 52% [product insert from Abbott, North Chicago, IL]), while some assays were specifically designed to measure only $25(OH)D_3$ (product insert from Roche, Indianapolis, IN). Reports have confirmed the variability of commercial immunoassays to detect $25(OH)D_2$ [163–166].

Immunoassay remains the predominant mode of measurement for 25(OH)D, although problems with equimolar recovery of the D_2 and D_3 metabolites remain an issue.

Almost all immunoassays show a high cross-reactivity with $24,25(OH)_2D$, which increases in concentration with increasing sun exposure; and as 25(OH)D increases and/or is metabolized to $1,25(OH)_2D$, this provides an increased supply of the two substrates for the 24-hydroxylase enzyme. Concentrations in the region of 10–15 nmol/L have been recorded for $24,25(OH)_2D$ in serum using GC–MS [167], with reported circulation levels of 10–15% that of 25(OH)D.

Concerns have been raised about a possible contribution to $1,25(OH)_2D$ measurement from other 1α-hydroxylated metabolites [168], and cross-reactivity for $1,25(OH)_2D_3$ 26,23-lactone, $1,24,25(OH)_3D_3$, and $1,25,26(OH)_3D_3$ has been demonstrated in both the Diasorin and IDS assays [169].

There have been major advances in semi automation and full automation of immunoassays utilizing nonradioactive tracers, which have been incorporated into both specialist-dedicated immunoassay systems.

4.2. Methods involving chromatographic separation

Liquid chromatography–tandem MS (LC–MS/MS) is regarded as the gold standard method for measurement of serum 25(OH)D concentrations due to its inherent analytical sensitivity and specificity [170–174], but the approach is equally powerful to detect other relevant vitamin D metabolites as well, either in single or multiple analyte format. The simultaneous measurement of vitamin D metabolites may provide better understanding of vitamin D metabolism in health and disease and predict which metabolite, or even combination of metabolites, may be the best indicators of vitamin D status.

Chromatography-based methods for vitamin D metabolite analysis are less susceptible to matrix effects than immunoassays. LC separations with subsequent detection, either by molecular absorption or molecular emission, have now largely been replaced by LC–MS/MS. This last approach has the advantage of selectivity, accuracy, and precision in analytical measurements as well as multiplexing capabilities, features that often greatly exceed those of immunoassays. LC–MS/MS is an approach that requires relatively expensive hardware and technical expertise operating in a batch-wise. Accurate and sensitive measurement requires optimization of many steps including sample preparation, calibration, chromatographic separation mode, choice of internal standard, ionization and transition selection for mass spectrometric detection [175]. Among the various ionization modes, ESI and APCI are the most commonly used techniques in MS vitamin D analysis, with minor use of atmospheric pressure ionization. There are reportedly over 50 vitamin D metabolites [176], apart from

numerous chemically synthesized analogs [177] and these must also be considered potential sources of assay interference. Most of the existing LC–MS/MS methods have adequate sensitivity for the measurement of 25(OH)D_3, 25(OH)D_2 and their respective C3-epimers, but the improvement in sensitivity required to include measurement of other relevant vitamin D metabolites that circulate at much lower concentrations is not always possible.This is the case with quantitation of 1,25$(OH)_2$D, which is present in serum at extremely low concentrations. The co-existence of many other higher abundant vitamin D metabolites that can interfere in 1,25$(OH)_2$D measurement constitutes an additional drawback. The LC–MS/MS platform has allowed development of methods of choice for 1,25$(OH)_2D_3$ analysis as it generates a higher selectivity in measurement when compared to immunoassays. Still, the accurate quantification of 1,25$(OH)_2D_3$ by LC–MS/MS is a challenge because of its low serum concentrations and lack of ionizable polar groups that result in poor ionization efficiency in ESI and APCI. Furthermore, specific care is needed to avoid potential interference from other dihydroxylated vitamin D metabolites, such as (3-epi) 24,25$(OH)_2D_3$, 23,25$(OH)_2D_3$, 25,26$(OH)_2D_3$ and 4β,25$(OH)_2D_3$, as they have the same molecular masses and fragmentation patterns. To enhance the detection response of the poorly ionizable compounds, derivatization strategies have been employed, mostly using Cookson-type triazoline-diones to react with the diene moiety of vitamin D, which enhances stability across the diene, and lends polar side chains to enhance ionization [9,178–182]. In some cases, mobile phase additives for adduct formation, such as ammonium, lithium or methylamine, have been used in order to further improve ionization efficiency [141, 182,183]. Recently, a new, commercially available reagent (Ampliflex™ Diene —AD—, AB Sciex, USA) was developed for derivatizing 1,25$(OH)_2$D that results in a 10-fold higher signal-to-noise ratio compared to PTAD [184]. The reaction product is optimized for MS/MS analysis due to its quaternary amine functional group and activated dienophile moiety. In contrast to PTAD, the fragmentation of the AD-1,25$(OH)_2D_3$ product is limited to several defined peaks with the quantifying SRM product containing the entire 1,25$(OH)_2$D structure, which results in different *m/z* values for the 1,25$(OH)_2D_3$ and 1,25$(OH)_2D_2$ product ions. This is beneficial in preventing isobaric cross talk between the two analytes. Also, the AD-1,25$(OH)_2$D metabolite products are more polar, but remain soluble in organic solvents. This hydrophilic property of the derivatization reaction products allows for the use of more rapid LC separation techniques [184]. Improvements in sample preparation have come from inmunoaffinity extraction(IAE) allowing analyte enrichment and removal of isobaric interferences and matrix effects present in patient serum [181,182,185].

The evolution of the chromatographic methods, mainly based on the type of the coupled detector, for determination of vitamin D and its metabolites can be shown through the contributions of the authors' research team. As commented before, the first methods were based on LC–conventional molecular absorption detector with no innovation in the separation–detection step, but in sample preparation [32–36]. Keeping the same chromatographic

separation, improvement by post-column derivatization and use of a conventional fluorimetric detector [37] or a laser-induced fluorescence detector [38] was achieved. The coupling of a DAD to the LC [40–43] avoided the derivatization step and provided similar sensitivity with drastic reduction of sample volume and automation of sample preparation [43], as a prelude of the optimization and application of the LC–MS/MS platform [44–48].

All the previous contributions of the authors' group established the basis for the recent improvements on sample collection and preparation [142], on stability of the target compounds [54], the use of SIL-ISs to support quantitative analysis and DEQAS samples for external validation of the method [49], and incorporation of two-dimensional LC to MS/MS for inclusion of the C3-epimer-25(OH)D in the analysis of vitamin D metabolites [143]. Thus, the way for massive application of the final method to the determination of the target compounds in serum or plasma samples from different cross-sectional and longitudinal studies was paved [186].

4.3. The use of SIL-ISs and validation in LC–MS methods to improve the determination of vitamin D metabolites

SIL-ISs are essential for compensating for ionization suppression or enhancement effects and correction of extraction losses during sample preparation.

Isotope dilution-electrospray LC–MS/MS methods performed on ''bench top'' analyzers became popular in the mid-2000s with protein precipitation of the sample, LLE, short run times, and computer processing of chromatograms contributing to higher throughput and ease of use in the determination of vitamin D and its metabolites [187,188]. Deuterated 25(OHD_2 and D_3 internal standard material improves accuracy and verifies recovery, thereby reducing the problem of ion suppression [189].

Isotope dilution LC–MS/MS is currently considered the gold standard method for 25(OH)D measurement, being able to simultaneously quantitate 25(OH)D_2 and 25(OH)D_3, with summation of the two values resulting in total 25(OH)D. A review of the International Vitamin D External Quality Assurance Scheme (DEQAS) results for the LC–MS/MS group highlights the spread of results generated by these methods. While the majority of the methods (70–75 %) are positively biased against the all-laboratory trimmed mean (ALTM), some are close to the mean (15–20%) or negatively biased depending on the 25(OH)D concentration measured (5–10%). There has also been concern raised regarding the presence of the 3-epi-25OHD epimer of 25(OH)D, which, because of the achiral nature of LC–MS/MS, cannot be separated from 25(OH)D by the majority of current methods. The presence of an epimer may increase the total 25(OH)D concentration measured by LC–MS/MS methods compared to immunoassays.

In 2009, the National Institute of Technology (NIST) released SRM 2972, ethanolic

25(OH)D3 and 25(OH)D2 calibration solutions to improve standardization of vitamin D assays. SRM 2972 has now been replaced by SRM 2972a 25(OH)D calibration solutions, which contain two ethanolic solutions of 25(OH)D3 as well as single solutions of 25(OH)D2 and 3-epi-25(OH)D3 [190].

5. Foreseeable/Desirable Future of Vitamin D Analysis

Since vitamin D and its metabolites have been found to be related to many acute and chronic biological disorders (cardiovascular diseases, cancer, diabetes, fractures, depression and respiratory tract infections, among others) their analysis has become of great interest. Generally, the total and free concentrations of vitamin D metabolites are estimated but free circulating metabolites are technically more complex to quantitate owing to their low concentration. In addition, diseases associated with vitamin D deficiency are usually related to concentrations even lower than normal levels.

One of the major advantages of LC–MS/MS assays is the capability of measuring multiple vitamin D metabolites simultaneously. While most current LC–MS/MS methods offer good results for relatively abundant vitamin D metabolites such as 25(OH)D, they do not provide the same performance for dihydroxymetabolites such as $1,25(OH)_2D_2$ or $24,25(OH)_2D_2$. This situation should change in the future by focusing the development/improvement of the methods on achieving more sensitive and selective quantitation [174].

Another critical aspect of great interest deals with the wide range of compounds resulting from vitamin D metabolism. The present evidences of the relationship between a number of biological disorders and the concentration, or concentration ratios, of low concentrated metabolites make mandatory their accurate determination to establish the suspected influence of given metabolites on the biological state of the organism with respect to the disease [50]. Either the intake of the given metabolites or the modification of the metabolic pathways to increase their concentration in the patient could be a medium-term objective after the short-term development of the appropriate analytical methods for their accurate measurement.

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