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Abstract

This chapter provides an overview of versatile and efficient chemical syntheses of vitamin D derivatives by application of either linear or convergent synthesis approaches. Synthesis of the most relevant naturally occurring vitamin D metabolites and their deuterated counterparts to use as calibration and reference standards in LC-MS/MS assays is also shown. The chapter then summarizes the most important mass spectrometric approaches to quantify important vitamin D metabolites in human biofluids. In addition, new developments are described that are aimed at the pathobiological interpretation of the measured vitamin D metabolite distributions in various human diseases.

Keywords: vitamin D deficiency, biomarkers, low abundant vitamin D metabolites, assay development, LC-MS/MS, diagnosis, disease risk prediction

1. Introduction

Vitamin D is mostly known for its role in the regulation of calcium and phosphorous homeostasis [1–3]. Consequently, vitamin D deficiency may cause various disorders related to bone mineralization [4]. Drugs based on vitamin D analogs are commonly used to treat bone diseases (osteoporosis, osteomalacia, and rickets) or psoriasis. More recently, it has been
suggested that vitamin D deficiency is also connected to a wide range of other diseases beyond bone mineralization, such as diabetes, autoimmune diseases, cardiovascular diseases, and cancer, as various clinical and epidemiological studies have shown [5–9]. However, the development of drugs for treatment of these diseases based on appropriate vitamin D analogs has mostly failed, either due to their rapid metabolic clearance or their calcemic effects. Vitamin D analogs are usually hormonally active compounds with pleiotropic functions and their levels in the body are strictly regulated by the hormonal system. In cases of oversupply, they are enzymatically degraded to avoid harmful effects such as calcemia, leading mainly to inactivation and conversion into water soluble degradation products suitable for renal clearance. Consequently, prevention of vitamin D deficiency rather than therapy of a vitamin D-related disease is a promising approach. Due to low concentration and short half-life of the active metabolite, 1α,25-dihydroxyvitamin D₃ (1,25(OH)₂D₃, calcitriol) (4) in serum, the biosynthetic precursor 25-hydroxyvitamin D₃ (25(OH)D₃) (3) is usually measured as a marker of vitamin D status. Additionally, more than 50 naturally occurring but low abundant vitamin D metabolites of mostly unknown physiological function have been identified. Common assays, particularly ELISA and radio-immunoassays (RIA), are often restricted to 25(OH)D₃ because of sensitivity and specificity limitations [10–12]. Fortunately, recent mass spectrometry advances have permitted reaching deeper into the metabolic cascade of vitamin D, including low abundant species [13–17]. Consequently, a wide variety of naturally occurring metabolites of potential biological activity can be analyzed simultaneously by liquid chromatography-tandem mass spectrometry (LC-MS/MS), which is currently considered the “gold standard” technique. However, this method has suffered in the past from the limited availability of the required vitamin D metabolites needed as calibration and reference standards. Consequently, as a prerequisite, all metabolites of interest have to be available for chemical synthesis, as described in this chapter.

2. Metabolism of vitamin D

Vitamin D₃ (2) is mainly synthesized in the skin from 7-dehydrocholesterol (1) by UV irradiation and subsequent thermal isomerization of the intermediate product previtamin D₃. Vitamin D₃ is then hydroxylated in the liver to 25(OH)D₃ (3), followed by further oxidation in the kidneys to 1,25(OH)₂D₃ (calcitriol) (4), which is usually considered the biologically active metabolite (Scheme 1). Calcitriol 4 is metabolized via CYP24 to other oxidative products, mainly 1α,24(R),25-trihydroxyvitamin D₃ (1,24,25(OH)₃D₃) (5), followed by subsequent enzymatic oxidation and degradation of the carbon side chain to water soluble calcitroic acid [1, 18] for final clearance from the body. 25(OH)D₃ (3) is degraded in an analogous manner via 24(R),25-dihydroxyvitamin D₃ (24,25(OH)₂D₃) (6). Alternatively, 25(OH)D₃ (3) and 1,25(OH)₂D₃ (4) can be metabolized to their corresponding 26,23-lactones (7,8) [19]. Vitamin D metabolites also have the potential to be metabolized through a C-3 epimerization pathway, leading to C-3-epi-metabolites such as 9-12, with an inversion of the stereogenic center at position C-3 [20, 21]. These reactions lead to products that are believed to be inactive. Since high concentrations of 3-epi-25-hydroxyvitamin D₃ (3-epi-25(OH)D₃) (10) were found in infants [22], it was initially
assumed that this pathway is only a consequence of an immature vitamin D metabolism. It has also been shown, however, that the epimerization pathway is favored if, for any reason, side chain degradation by CYP24 is inhibited [23].

**Scheme 1.** Metabolic pathways of vitamin D.

Finally, the corresponding metabolites of vitamin D$_2$ (13) also have to be considered [18], because food from plant origin (in particular mushrooms) and food supplements may contain vitamin D$_2$. Vitamin D$_2$ metabolites have similar physiological function as compared to their corresponding vitamin D$_3$ analogs, although their potency is apparently lower [24]. A detailed comparison of the metabolism of vitamin D$_2$ and D$_3$ [18] reveals that 25(OH)D$_3$ (3) and 1,25(OH)$_2$D$_3$ (4) are preferentially metabolized at their side chains, particularly at C-23, C-24, and C-26, leading to metabolites susceptible to further oxidation, which is similar for vitamin D$_2$, even though there are slight differences as a consequence of the C-22/23 carbon double bond and a methyl group at C-24. Thus, C-24 hydroxylation of vitamin D$_2$ may occur initially, leading to 24-hydroxyvitamin D$_2$, following by C-1 hydroxylation in the kidneys, giving 1,24-dihydroxyvitamin D$_2$, which is subsequently oxidized in the side chain. Furthermore, 1,25-
dihydroxyvitamin D<sub>2</sub>, formed in an analogous manner to its D<sub>3</sub> counterpart, may be metabolized by side chain oxidation either to C-1,24,25 or C-1,25,26 trihydroxylated vitamin D<sub>2</sub> again followed by further side chain oxidation. Each of these both pathways again leads to calcitriolic acid as final metabolite.

3. Synthesis of low abundant vitamin D metabolites

The commercial unavailability of many relevant vitamin D<sub>3</sub> and D<sub>2</sub> species has limited the scope of LC-MS/MS assays in the past. For use as reference standards, vitamin D<sub>3</sub> and D<sub>2</sub> metabolites, as well as their corresponding stable isotopes (labeled with $^2$H (D) or $^{13}$C), have to be synthesized by application of a versatile and cost-effective methodology. A large variety of chemical syntheses of vitamin D derivatives has been developed in the last few decades by several academic and commercial groups [1, 25–27], mainly with the aim of synthesizing new analogs for drug discovery and development purposes. These efforts have resulted in a large number of more than 3000 synthesized compounds [28]. Critical evaluation of these methods reveals, however, that only a few of them are suitable to reproducibly generate metabolites of interest in gram quantities at reasonable costs within short time frames. In this chapter, we will review some of the more suitable strategies that have been successfully applied and optimized in our laboratory.

The synthesis of deuterated vitamin D<sub>3</sub> and D<sub>2</sub> metabolites is mostly accomplished using the same procedures as those developed for nondeuterated metabolites.

The biosynthetic and technical synthesis (starting from an appropriate steroid precursor (analog to 1) is not favorable, due to low yields reached in the photochemical ring-opening reaction. It usually fails if a 1-hydroxy substituent is present. By far the most suitable methods for synthesis of vitamin D<sub>3</sub> and D<sub>2</sub> metabolites start with readily available Vitamin D<sub>2</sub> (13). Two alternative strategies are applied, either by keeping the vitamin D skeleton intact during the course of the reaction sequence (linear synthesis) (Scheme 2), or by first cleaving the molecule to obtain the A-ring and the CD-ring building block, and subsequently reconnecting both molecules after separate appropriate chemical modifications (convergent synthesis) (Scheme 3) [1, 26, 27, 29, 30].

3.1. Linear synthesis of vitamin D metabolites

Following a linear synthesis (Scheme 2), the cis-diene moiety of vitamin D<sub>2</sub> (13) is protected by sulfur dioxide after silylation of the hydroxyl group at C-3 as a tert-butyldimethylsilyl ether, leading to the SO<sub>2</sub> adduct 14. Labeling with deuterium at C-6,19,19 can be carried out conveniently by treatment with D<sub>2</sub>O or deuterated alcohol (e.g., isopropanol). Finally, thermal removal of SO<sub>2</sub> and photochemical isomerization leads to threefold deuterated vitamin D<sub>2</sub> (d-13). This simple and convenient method to obtain C-6,19,19 deuterated metabolites [31] can also be applied to a wide variety of other vitamin D<sub>3</sub> and D<sub>2</sub> derivatives. In order to obtain metabolites of the corresponding C-3-epi series of vitamin D<sub>3</sub> or D<sub>2</sub> metabolites, the configuration of the C-3 OH group of vitamin D<sub>2</sub> (13) can be epimerized under the so-called “Mitsunobu conditions”
[32], by treatment with an aromatic acid and an azodicarboxylate, resulting in the corresponding ester 15 with concomitant inversion of the stereogenic center at C-3 (orientation of the substituent has changed from β to α). The ester is finally cleaved by saponification with potassium hydroxide or by reduction with lithium aluminum hydride to afford C-3-epi vitamin D$_2$ (16), which can be used to synthesize a wide variety of C-3 epimers, including 3-epi-25-hydroxyvitamin D$_3$(3-epi-25(OH))D$_3$ (10, d-10) and 3-epi-1α,25-dihydroxyvitamin D$_3$(3-epi-1,25(OH))$_2$D$_3$ (11, d-11). Yields are only moderate due to the favored formation of a C-3,4,5,6,7,8 all-trans triene system by elimination. To further proceed with the synthesis, the C-22/23 double bond in the side chain of SO$_2$ adduct 14 is cleaved by ozonolysis, leading to alcohol 17 after reductive workup with sodium borohydride, which can be used to add a wide variety of modified side chains as appropriate. For instance, 17 can be converted to an iodide, which is coupled in a nickel-zinc-mediated reaction with ethyl acrylate after prior removal of the SO$_2$ protective group to afford 18. At this stage, an allylic oxidation by selenium dioxide can be carried out, leading to a 1α-hydroxy series of metabolites, such as 19 as appropriate precursor. Silylation of the newly generated hydroxyl group, followed by Grignard reaction with methylmagnesium bromide, leads to 1α,25-dihydroxy metabolites. If a deuterated Grignard reagent, labeled with 3 deuterium atoms at their methyl groups, is employed, the corresponding sixfold deuterium labeled metabolite is obtained, containing 3 deuterium atoms at C-26 and C-27, respectively. Finally, the C-5/6 double bond has to be isomerized photochemically, and the silyl protective groups have to be removed, ending up with nondeuterated or deuterated 25(OH)D$_3$(3, d-3) or 1,25(OH)$_2$D$_3$(4, d-4) [33], as shown in Scheme 2. If 3-epi-vitamin D$_2$ (16) is employed in this sequence, 3-epi-25(OH))$_2$D$_3$ (10, d-10) is obtained. 3-epi-1,25(OH))$_2$D$_3$(11, d-11) is obtained, if 1α-hydroxylation by selenium dioxide was carried out, and the resulting C-1-OH-group is epimerized by oxidation and final reduction.

Vitamin D$_2$ metabolites are synthesized by application of analogous strategies as applied for their corresponding D$_3$ counterparts, although they are more challenging to synthesize, for two reasons: vitamin D$_2$ metabolites contain an olefinic double bond in C-22/23-position, which has to be arranged in trans geometry during the course of a suitable coupling reaction of a CD-ring precursor and an alternated side chain building block by olefination. Most synthetic olefination methods yield a mixture of cis and trans double bonds, leading to product mixtures that are difficult to separate due to their similar polarity. If the reaction conditions are too basic, C-20 epimerization can also occur, leading to even more complex product mixtures. Additionally, vitamin D$_2$ metabolites contain a chiral methyl group in C-24 position that has to be ranged in a defined configuration. When a suitable building block bearing this methyl group is employed in the synthesis, epimerization of the chiral center may occur under basic reaction conditions, along with the risk for epimerization at C-20 in the coupling reaction. Thus, alcohol 17 is converted to its corresponding phenylsulphone, which is coupled by olefination with aldehyde 20, a method originally invented by Julia [34]. Note that 20 can be synthesized in a few routine steps from commercially available (S)-(−)-3-hydroxyisobutyric acid methyl ester, which is submitted to a Grignard reaction with methyl-magnesium bromide. Analogously to the synthesis of the other sixfold deuterated metabolites labeled at C-26/27, the addition of deuterated Grignard reagent in the course of the reaction leads to sixfold deuterated vitamin
D$_2$ metabolites. Finally, the double bond at C-5/6 of 21 has to be photochemically isomerized and the silyl protective groups have to be removed, leading to nondeuterated or deuterated 25(OH)D$_2$ (22, d-22) [35].

3.2. Convergent synthesis of vitamin D metabolites

Alternatively, a convergent approach can be applied (Scheme 3), which is more versatile than a linear synthesis and allows for more harsh reaction conditions and wider scope of suitable substrates and reagents. In a classical and widely applied strategy, vitamin D$_2$ (13) is submitted to ozonolysis, leading to Inhoffen-Lythgoe diol 23 and allylic alcohol 24 after reductive workup with sodium borohydride. After appropriate chemical modification of the CD-ring precursor, this compound is coupled with an A-ring building block. Two strategies can be distinguished. In the most common approach, invented by Lythgoe and further developed by the Hoffmann-La Roche group [36], allylic alcohol 24 is converted via an allylic chloride 25 in diphenylphosphine oxide 26, which is coupled with a C-8 ketone by a Wittig-Horner olefination. The A-ring diphenylphosphine oxide 26 can be coupled before or after appropriate alteration of the side
chain at the CD-ring building block. To employ an A-ring diphenylphosphine oxide such as 26 is favourable, if no further substituents except a C-3 OH group and a C-10 exo-methylene group in the A-ring are needed, as it is already present in vitamin D$_2$ (13). As example for an efficient application of this strategy, the synthesis of 24,25(OH)$_2$D$_3$ (6) and its deuterated counterpart (d-6), their 3-epi-analogs (9/d-9) and 1α-analogs (5/d-5), is shown in Scheme 3 [37]. Here, diol 23 is converted to its corresponding iodide, which is coupled with enone 27 (obtained in a few routine steps from (R)-(-)-lactic acid using a zinc-copper-catalyzed reaction mediated by ultrasound, leading to 28. By Grignard reaction of 28 with nondeuterated or deuterated methyl-magnesium bromide, 29 or d-29 are obtained. The 1,2-diol moiety is protected as isopropylidene ether, followed by oxidation with pyridinium chlorochromate to obtain ketone 30/d-30, which can be coupled with diphenylphosphine oxide 26 to obtain d-24,25(OH)$_2$D$_3$ (d-6) after removal of silyl- and isopropylidene protective groups. To access the corresponding C-3-epi-analog, vitamin D$_2$ is first bis-hydroxylated at C-7/8 using potassium permanganate. Inversion of the stereogenic center at C-3 under “Mitsunobu-conditions” using picolinic acid leads to the corresponding C-3-epi-triol 31. The yields of this reaction are much higher as compared to the synthesis of 16 used in the linear synthesis, because an elimination reaction leading to a favored C-3,4,5,6,7,8-all-trans system is avoided [38]. Silylation and cleavage of the diol moiety of triol 31 by lead tetraacetate and reductive workup with sodium borohydride lead to the CD-ring fragment 32 and C-3-epi allylic alcohol 33, which can be used after conversion to the corresponding diphenylphosphine oxide 34. Note that 34 is coupled to d-30 to obtain d-3-epi-24,25(OH)$_2$D$_3$ (d-9) or its corresponding nondeuterated counterpart 9. 3-epi-diphenylphosphine oxide 34 can be used as a versatile A-ring building block to be employed

Scheme 3. Convergent synthesis of 24,25(OH)$_2$D metabolites. TBDMSCl: tert-butyldimethylsilyl chloride, O$_3$: ozone, NaBH$_4$: sodium borohydride, I$_2$: iodine, Cu: copper, Zn: zinc, D$_2$CMgBr: deuterated methylmagnesium bromide, Me$_2$CO: acetone, PCC: pyridinium chlorochromate, KMnO$_4$: potassium permanganate, DIAD: disopropyl azodicarboxylate, NCS: N-chlorosuccinimide, LiPPh$_2$: lithium diphenylphosphide, H$_2$O$_2$: hydrogen peroxide. BuLi: n-butyllithium, Bu$_4$NF: tetra-n-butylammonium fluoride, Pb(OAc)$_4$: lead tetraacetate, SeO$_2$: selenium dioxide.
in a Wittig-Horner reaction for the synthesis of a wide variety of 3-epi vitamin D₃ and D₂ metabolites, by connection with appropriate CD-ring building blocks with modified side chain, as shown for the synthesis of 3-epi-24,25(OH)₂D₃ (9, d-9) as representative example. Finally, permanganate oxidation of vitamin D₂ (13) leads to triol 35, which is converted in a few routine steps, including a stereoselective 1α-allylic oxidation with SeO₂ via 36 to diphenylphosphine oxide 37, which is then coupled with d-30 to afford 1,24,25(OH)₃D₃ (5/d-5).

Two other low abundant metabolites, 23(S),25(R)-dihydroxyvitamin D₃ 26,23-lactone 7 and 1α-1,23(S),25(R)-trihydroxyvitamin D₃ 26,23-lactone 8, are synthesized in an analogous manner [29] (Scheme 4), by coupling a CD-ring ketone carrying an appropriate side chain with diphenylphosphine oxide 26 or 37. Here CD-ring-building block 32 obtained from vitamin D₂ 13 is used, which is converted to aldehyde 38. In order to prepare an appropriate modified side chain, isopentenol 39 is converted using a few routine steps to sulfone 40, which is then coupled with 38 by a Julia olefination to afford 41. Removal of the isopropylidene protective group, oxidation to an unsaturated α-hydroxy carboxylic acid and subsequent iodolactonization leads to ketone 44 via 42 and 43, which is coupled with diphenylphosphine oxide 26 or 37, respectively, to give 7 and 8.

Scheme 4. Convergent synthesis of 23,26(OH)ₓ-lactone metabolites. TBDMS: tert-butyldimethylsilyl chloride, O₃: ozone, LDA: lithium diisopropylamide, Na: sodium, Hg: mercury, PPTS: pyridinium p-toluenesulfonate, DMSO: dimethyl sulfoxide, Py: pyridine, SO₂: sulfur dioxide, I₂: iodine, KOH: potassium hydroxide, Bu₃SnH: tributyltin hydride, Bu₄NF: tetra-n-butylammonium fluoride, PCC: pyridinium chlorochromate, BuLi: n-butyl lithium.

3.3. Convergent Palladium-catalyzed synthesis of vitamin D metabolites

The A-ring building block is preferably synthesized de novo, if further substituents apart from a C-3-OH group and a C-10 exo methylene group in the A-ring (already present in vitamin D₂) are needed. This is particularly valuable for synthetic analogs containing substituents at C-2, which were developed in the past as new drugs [30]. However, natural metabolites may
also contain additional substituents in the A-ring, such as 4β,25-dihydroxy vitamin D₃ [39]. In these cases, Pd catalyzed tandem reactions can be applied (Scheme 5). Initially invented by Trost [40], an acyclic enyne 46 is coupled with a CD-ring vinyl bromide 47 obtained by Peterson olefination from the corresponding ketone. Closure of the A-ring and its connection with CD-ring is preferably carried out in one pot. Alternatively, the corresponding triflate 48 can be employed in a Zn-mediated reaction, which was invented by Negishi [41]. Additionally, CD-ring alkene boranates 49 can be used via an analogous coupling method originally invented by Suzuki [42]. Finally, a cyclic A-ring enyne, such as 50, can be coupled with CD-ring triflate 51, a coupling method invented by Sonogashira [43]. In all these cases, the CD-ring vinyl bromide 47, boranate 49, or triflate 51 are usually carrying the modified side chain already, before coupling with the acyclic or cyclic A-ring enyne. A major drawback of these methods is the fact that synthesis of acyclic or cyclic enynes or other A-ring building blocks not derived from vitamin D₂ involves many synthesis steps, and in some cases separation of diastereomeric mixtures is also necessary to obtain enantiomerically and diastereomerically pure product. However, the suitable starting materials can usually be obtained from the natural chiral pool (i.e., terpenes such as carvone [44], malic acid [45], quinic acid [43], or carbon hydrates such as D-glucose [46] or D-xylose [47]), which are usually readily available. A wide range of substrates are tolerated. Consequently, these advanced strategies involving Pd catalyzed reactions have widely been applied in recent years and are by now well established.

Scheme 5. Convergent Pd-catalyzed synthesis of vitamin D metabolites via de novo-A-ring synthesis. Pd: palladium, ZnCl₂: zinc chloride.
4. LC-MS/MS assays for vitamin D metabolites

In the second part of this chapter, an overview of the mass spectrometric analysis of vitamin D metabolites in biological samples is presented. Because of the chemical nature of these secosteroidal molecules, liquid chromatography-tandem mass spectrometry (LC-MS/MS) currently provides the optimum analytical platform for analysis of vitamin D metabolites. While mass spectrometry assays are often not as rugged and more expensive than most non-mass spectrometric assays (in particular immunoassays), they provide the ability to capture multiple metabolites simultaneously at very low concentration levels. Immunoassays measure the 25(OH)D$_3$ and 25(OH)D$_2$ metabolites only and have limitations with respect to detection sensitivity as well as selectivity and specificity issues.

In most published assays, the metabolites are determined from serum or plasma matrices. Other biological matrices have rarely been used, although vitamin D metabolites have successfully been quantified in saliva [48] and various soft tissues [49]. In the following sections, we discuss the requirements and characteristics of LC-MS/MS of vitamin D metabolites; that is, chromatographic separation, ionization, m/z analysis, interferences and accuracy issues, multimetabolite screening applications, and the clinical role of vitamin D fingerprinting methods.

4.1. Liquid chromatographic separation of vitamin D metabolites

Due to the hydrophobic structure, vitamin D metabolites are generally easily separated on reversed-phase liquid chromatography stationary phases (e.g., octadecyl C$_{18}$) materials utilizing hydrophobic interactions. The vitamin D metabolites generally elute in the order trihydroxylated < dihydroxylated < monohydroxylated metabolites, that is, for vitamin D$_3$-related molecules, the order of chromatographic retention is 1,25(OH)$_2$D$_3$ < 25(OH)D$_3$ < D$_3$. Between corresponding D$_2$ and D$_3$ analogs, the D$_2$ metabolites elute marginally later than the D$_3$ versions. For the two important isomers of dihydroxylated vitamin D metabolites, 24,25(OH)$_2$D$_3$ and 1,25(OH)$_2$D$_2$, the order of retention is 24,25(OH)$_2$D$_3$ < 1,25(OH)$_2$D$_2$.

Possibly more interesting—from a chromatography point of view—are the biochemically formed isomers after stereochemical reversal ($\beta\rightarrow\alpha$) at C-3; for example, the epimers 25(OH)D$_3$ and 3-epi-25(OH)D$_3$. The subtle diastereomeric differences between these species can be distinguished using specialized columns, such as combined C18/chiral [50], pentafluorophenyl (PFP) [51–53], and cyano (CN) [54–57].

4.2. Ionization of vitamin D metabolites for mass spectrometric analysis

Mass spectrometric determination of vitamin D metabolites using liquid chromatography-mass spectrometry (LC-MS) is not trivial because of the structural limitations that the analytes provide with respect to attaching a charge to the molecules. Gas chromatography-mass spectrometry techniques have been used in the past for qualitative analysis and structure determinations, but these methods have been almost completely replaced by modern LC-MS
methods. While analysis of transformation products such as vitamin D sulfates is relatively easy by LC-MS as the metabolites can be simply analyzed as deprotonated molecules, ionizing the relatively nonpolar vitamin D metabolites is not straightforward. For LC-MS, electrospray ionization (ESI) or atmospheric pressure chemical ionization (APCI) are the most common ionization techniques, which usually result in the formation of [M+H]+ ions for most biological molecules. APCI has been successfully applied to steroids, as gas-phase chemical ionization often efficiently transfers a proton to these types of molecules. ESI usually relies on a charging mechanism in the liquid phase and one would therefore expect ESI to be less efficient than APCI for vitamin D metabolites. In reality this is not the case, however, and both techniques have equally been applied to vitamin D analysis, with the analytical figures of merit being quite similar between the two ionization techniques. There appears to be a slight trend toward application of ESI rather than APCI in recent years.

An important, often neglected consideration in the choice of ionization source is the (usually detrimental) impact of coionized sample components that coelute with vitamin D metabolites from the LC column. Here, ionization suppression effects caused by these coeluting molecules may impact the outcome even stronger than the differences seen in ionization efficiencies between ESI and APCI [58, 59]. However, no systematic comparison of ionization efficiency/ionization suppression effects between ESI and APCI has been performed yet. Furthermore, coionization of coeluting components also leads to the formation of isobaric interferences when sample matrices such as plasma or serum are analyzed, which will be discussed in more detail below.

Another useful ionization technique is atmospheric pressure photoionization (APPI). This technique is very common in fields such as petroleum analysis, but surprisingly, it is virtually unknown in the clinical community. To our knowledge, only one study has systematically compared APPI with APCI and shown that APPI generated significantly higher ion currents for 25(OH)D₃ than APCI [60]. A second study applied APPI to quantification of 25(OH)D₃ without comparison to ESI or APCI [61].

Finally, another option to overcome problems of ionization efficiency and resulting problems with detection sensitivity is derivatization of vitamin D metabolites, to convert them into better responding transformation products. Such procedures are now quite common in the vitamin D analytical field; they comprise introduction of a chemical group that is readily ionized or is permanently charged. Cookson-type triazoline-diones and triazoline-dione-related reagents (e.g., 4-phenyl-1,2,4-triazoline-3,5-dione [PTAD]) are most often applied, which utilize the reaction of the reagent’s dienophile with the cis-diene group at C-5/C-6 and C-10/C-19 of vitamin D (Diels-Alder [4+2] reaction) (Scheme 6).

Several promising PTAD assays have been described in the literature [39, 49, 62–64]. The advantage of using the cis-diene moiety is that all vitamin D metabolites contained in the sample are converted when the reagent is present at sufficient concentrations (i.e., in large excess quantities) as all relevant vitamin D metabolites possess this structural motif. Ionization efficiency of 25(OH)D-D-PTAD has been reported to be 100-fold higher than the nonderivatized molecules in ESI mode [65]. A structural variation in PTAD includes a permanently charged quaternary ammonium moiety, which enhances detection sensitivity even further [66].
reagent is commercially available. A recent chemotyping assay for multiple vitamin D metabolites has shown detection limits in the fg/mL range for vitamin D metabolites from human serum (see discussion below).

![Scheme 6. Diels-Alder [4+2] derivatization of vitamin D metabolites using PTAD.](image)

### 4.3 Mass analyzers for vitamin D analysis

Most clinical applications for vitamin D utilize quadrupole-based mass analyzers with low resolving powers. Because these mass spectrometers measure \( m/z \) signals at “unit mass resolution,” they cannot be operated in full scan mode for the accurate quantification of vitamin metabolites when complex sample matrices such as serum are analyzed. Serum contains multiple components of identical nominal molecular mass to vitamin D metabolites, several of which have been shown to coelute during High Performance Liquid Chromatography (HPLC) [67]. The coeluting metabolites then generate so-called “isobaric” ions in the mass spectra after ESI, which cannot be distinguished from the vitamin D target molecules using low resolution MS. Therefore, tandem mass spectrometry (MS/MS) is implemented, which activates the ionized species further and forces them to undergo collision-induced dissociation (CID) for generating structure-specific product ions. The metabolite-specific product ions are then measured and their response used for quantification of the target metabolites.

By far the most common low resolution tandem mass spectrometer for vitamin D analysis in clinical laboratories is the triple quadrupole (QqQ) instrument. In a QqQ mass spectrometer, the \([M+H]^+\) ions of vitamin D metabolites are selected in the first quadrupole, fragmented in a quadrupole collision cell, and the products analyzed in the final quadrupole mass analyzer. The acquisition mode that is almost always applied is the so-called selected reaction monitoring (SRM) mode. In this mode, the \([M+H]^+\) precursor ion is isolated in Q1 (e.g., \( m/z \) 401 for 25(OH)D₃), dissociated in q2, and one specific product ion selected in Q3 (e.g., \( m/z \) 383). The quality of the precursor/product ion selection determines the selectivity of analysis; that is, the product ion structure should ideally directly reflect the chemical structure of the chosen precursor ion for maximum specificity. For vitamin D metabolites, the selection of an appropriate product ion is surprisingly difficult because of some inherent structural limitation of the precursor structure, leading primarily to simple dehydration reactions at low collision energies, which turn into complex mass spectra at higher collision energies (Figure 1) [68].
Unfortunately, because of multiple, overlapping series of fragmentation reactions, leading to “picket fence” type product ion spectra, fragment ions for vitamin D metabolites in the diagnostic m/z range often resemble each other.

Figure 1. Collision-induced dissociation (CID) spectrum of the [M+H]+ ion of 1,25(OH)2D3 (spectrum after fully completed dehydration reactions after electrospray ionization and CID; m/z analysis by Fourier-transform ion cyclotron resonance, FTICR).

A few studies also report the use of a low resolution quadrupole ion trap (IT) [69, 70] in MS/MS mode for 25(OH)D3 and 25(OH)D2, but these instruments are rarely implemented in clinical environments. IT instruments are often not fit for purpose for quantitation of low abundant biological molecules, because of the large detrimental contributions of acquisition time overhead to the duty cycle, thus limiting detection sensitivity. There is also a general decline of this instrument type in modern mass spectrometry labs.

The most anticipated future development for vitamin D analysis is the introduction of high resolution mass spectrometry (HRMS), which is now firmly established in many other fields of modern mass spectrometry, in particular, in pharmaceutical applications and the proteomics field [71]. This trend is mostly due to the availability of robust quadrupole-quadrupole-time-of-flight (QqTOF) and orbitrap mass analyzers in recent years, which have transformed many analytical approaches to mass spectrometry. The use of mass defect as metabolite-specific property, for example, is now an integral part of many metabolite identification routines for drug metabolites [72]. While the number of applications of HRMS in the vitamin D field is still limited, the existing work has clearly demonstrated the potential of HRMS for vitamin D analysis. For example, orbitrap mass spectrometers in full scan [73, 74] and MS/MS [75, 76]...
modes have been applied successfully to the analysis of 25(OH)D$_3$ in human serum and analytical performance has been shown to be equivalent or better than triple quadrupole and immunoassays. The important role of HRMS in the separation of isobaric interferences will be shown below.

4.4. Interferences during LC-MS/MS

The LC-MS/MS analysis of vitamin D metabolites is affected by various sources of error, which can affect both precision and accuracy. As with any other LC-MS/MS analysis from biological samples, ion suppression by coeluting sample components or chemical modifiers from the sample preparation or chromatography can lead to reduced analyte signals. There are several options to assess whether or not ion suppression is present, which have been summarized in many review articles, e.g., by Matuszewski et al. [77]. Stable isotope standards of vitamin D metabolites (mostly deuterated analogs) can usually correct for accuracy errors from ion suppression effects, as long as it is guaranteed that protein binding for the isotope standard is the same as for the endogenous analyte, which requires implementation of a careful incubation routine [78]. Importantly, deuterated isotope standards are commercially available for most of the relevant vitamin D metabolites but unfortunately not for all.

A second important source of analytical error originates from isobaric noise; that is, endogenous or exogenous metabolites that coelute with the vitamin D analytes and erroneously contribute to the analytical signal, if unspecific ions are used for mass spectral analysis. This has recently been described in detail by Qi et al. [67], who clearly demonstrated the presence of multiple isobars of 25(OH)D$_3$ in human serum. The isobars have to be carefully removed in low resolution mass spectrometry, by application of appropriate MS/MS or ion mobility spectrometry routines [67]. A number of exogenous and endogenous molecules have been identified as relevant metabolites in serum samples [50, 67, 79].

Many of these interferences can be eliminated by application of high resolution mass spectrometry using sufficiently high resolving powers; for example, through implementation of orbitrap or Fourier-transform ion cyclotron resonance (FTICR) mass spectrometers. For example, Liebisch and Matysik demonstrated that the orbitrap MS instrument in their study was able to separate an isobaric interference of 25(OH)D$_3$ in the MS/MS domain; this interference was caused by fragmentation of the d6-25(OH)D$_3$ isotope standard [75].

Importantly, the issues relating to isobaric noise have only been studied for the 25(OH)D$_3$ analyte; other metabolites will likely be affected by similar interferences, the impact of which during quantification, in particular, in multimetabolite assays (see below), remains unknown.

4.5. Method accuracy and certified reference materials

The vitamin D analytical community is supported through the Vitamin D External Quality Assessment Scheme (DEQAS), a nonprofit organization that evaluates the performance of analytical assays of member laboratories for 25(OH)D$_3$ and 1,25(OH)$_2$D$_3$ [80] through round-robin analyses. DEQAS has clearly demonstrated that analytical performance of vitamin D analysis has greatly improved over the years between 1994 and 2009 [81].
The United States National Institute of Standards and Technology (NIST) provides certified standard solutions for 25(OH)D$_3$ and 25(OH)D$_2$. Furthermore, the NIST and the National Institutes of Health (NIH) Office of Dietary Supplements (ODS) have established the Vitamin D Metabolites Quality Assurance Program (VitDQAP), for interlaboratory comparison of measurement of 25(OH)D$_2$, 25(OH)D$_3$, and 3-epi-25(OH)D$_3$ in serum and plasma and provide method-appropriate control materials.

A number of commercial reference, calibration, and quality control materials are available from several companies that allow rapid implementation and validation of vitamin D analytical methodologies.

4.6. Vitamin D multimetabolite assays

One of the most important advantages of LC-MS/MS assays over clinical immunoassays is the ability to determine multiple vitamin D species independently and simultaneously. As a result, there are now several very capable LC-MS/MS assays described in the literature that provide the capability for profiling the most relevant vitamin D metabolites at the same time, within a single analytical run, and with sufficient dynamic range to allow measuring the required physiological levels, down to the picomolar range. The topic has recently been reviewed in detail and the interested reader is referred to Ref. [82]. Briefly, most of these multimetabolite assays utilize derivatization techniques that transform all vitamin D species into better responding analogs (vide supra). This procedure in turn permits analysis of both high and low abundant vitamin D species with similar analytical figures of merit [62, 83].

The simultaneous acquisition of all important metabolite levels then provides the possibility of using these vitamin D metabolite distributions (“chemotypes”) as complex diagnostic or prognostic biomarkers for correlation with disease phenotype or clinical outcome of treatment. A few examples for such correlations are summarized in the last section.

5. Vitamin D fingerprints (chemotypes) in clinical applications

A number of recent studies have gone beyond the usual determination of 25(OH)D$_3$—as marker for vitamin D status [84]—and 1,25(OH)$_2$D$_3$—for diagnosis of renal diseases, hypercalcemic syndromes, and disorders of 25(OH)D$_3$ metabolism [85]. These broader profiling techniques are aimed at discovering dynamic effects of metabolites and catabolites, which are located further downstream the 25(OH)D$_3$ metabolic cascade. Current studies highlight 24,25(OH)$_2$D$_3$ as important diagnostic marker, which was previously only considered a clearance product of vitamin D without activity. In fact, it has been shown that 24,25(OH)$_2$D$_3$ has crucial roles in bone metabolism [86] and renal diseases [87].

Capturing multiple vitamin D species and their dynamic changes allows for a better understanding of interindividual variations after vitamin D supplementation. Müller et al. recently demonstrated an inverse linear correlation between baseline 25(OH)D$_3$ and response to supplementation for patients with chronic liver disease [83]. The study also showed that lower
baseline 24,25(OH)₂D₃ levels were linked to larger changes of 25(OH)D₃ levels, and that those patients who exhibited greater response to vitamin D supplementation had lower levels of 3-epi-25(OH)D₃ (Figure 2).

Berg et al. implemented the ratio of 24,25(OH)₂D₃ and 25(OH)D₃ as a novel status marker for vitamin D [88]. Wagner et al. used the same ratio and demonstrated that it was predictive of 25(OH)D₃ response to supplementation [89]. Binkley et al. measured multiple vitamin D species and developed a model to describe interindividual variation of 25(OH)D₃ levels after supplementation [90]; the authors highlighted the role of absorption (as measured by the nonmetabolized vitamin D₃ species) and degradation (via the 24,25(OH)₂D₃ species) and presented a treat-to-target regime for tailored serum levels of 25(OH)D₃ [90].

Figure 2. Nonparametric correlations between baseline vitamin D metabolites and response to vitamin D supplementation for patients with chronic liver diseases after 6-month treatment: (a) the relative change in serum 25(OH)D₃ (in response to vitamin D supplementation) correlated inversely with baseline 25(OH)D₃ concentrations; (b) similarly, an inverse correlation between relative change in serum 25(OH)D₃ and baseline 24,25(OH)₂D₃ was observed; (c) baseline 24,25(OH)₂D₃ concentrations were nonsignificantly higher in women as compared to men; (d) patients with lower 3-epi-25(OH)D₃ concentrations at baseline tended to have a larger response to vitamin D supplementation (reprinted with permission from Ref. [83]).

Other important studies include the work by Bosworth et al. [87] and Stubs et al. [91], who utilized multimetabolite LC-MS/MS methods to characterize chronic kidney disease (CKD). Similarly, Duan et al. [64] studied patients with multiple sclerosis and observed comparable
levels of 25(OH)D3 in the healthy control subjects and the patients; however, serum levels for 1,25(OH)2D3 and 24,25(OH)2D3 were lower in patients than controls.

6. Conclusions

The availability of assays for simultaneous capturing multiple vitamin D metabolites combined with reliable techniques for synthesis of the required metabolite standard compounds makes accurate measurement of metabolite distributions and subsequent correlation with disease phenotype readily possible; the outcome of these strategies is expected to be useful for diagnosis and risk prediction for various diseases. It may also allow for specific supplementation strategies in the future, which consider patient-specific dosage requirements and use of selected vitamin D metabolites or analogs.

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